Can Walnut Serve as a Magic Bullet for the Management of Non-Alcoholic Fatty Liver Disease?

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Featured Application: Walnut can be a natural remedy for non-alcoholic fatty liver disease through a variety of components that can modulate the underlying etiologic mechanisms.

Abstract: Walnut contains many nutrients and bioactive components such as essential fatty acids, polyphenols, fiber, γ-tocopherol, folate, minerals, and vegetable protein, and has therefore been regarded as a natural functional food. Walnut-enriched diets have been demonstrated to be useful for heart health, cancer prevention, and metabolic disorders owing to their anti-oxidative and anti-inflammatory properties as well as for the maintenance of a healthy metabolism and immune function. Walnut extracts, either phenolic or lipid, also demonstrated the health effects in animal and cultured cell studies. More recently, the beneficial effects of walnut consumption on non-alcoholic fatty liver disease, which is a hepatic manifestation of obesity, hyperlipidemia, type 2 diabetes mellitus, and metabolic syndrome with substantial hepatic accumulation of triglyceride, have been proposed because walnut and a walnut-containing diet can modulate the etiologic mechanism such as ameliorating systemic and hepatic dyslipidemia, reducing lipotoxicity and inflammation, enhancing immune function, and maintaining gut microbiota balance. Through the extensive literature review we discuss the preventive roles of walnut in the development and progression of non-alcoholic fatty liver disease (NAFLD) and provide mechanistic insights into these effects.

Keywords: walnut; non-alcoholic fatty liver disease (NAFLD); non-alcoholic steatohepatitis (NASH); one-carbon metabolism; dyslipidemia; lipotoxicity; gut microbiota

1. Introduction

Nuts are natural food sources and are globally one of the most popular snacks or ingredients of a healthy diet. Walnut has a distinct composition compared with that of other nuts [1]. It is an excellent source of essential fatty acids such α-linolenic acid and oleic acid [2] and contains high levels of polyphenols, such as gallic acid, (+)-catechin, chlorogenic acid, and ellagic acid [3], in addition to being rich in fiber, and vitamins such as γ-tocopherol, folate, and vitamin B 6. Walnut also contains minerals such as copper, chromium, iron, and zinc and plant compounds such as phytic acid and melatonin. Furthermore, walnut is a healthy source of vegetable proteins. Thus, walnut is a natural functional food and the incorporation of walnut into the human diet is highly desirable for the prevention of chronic diseases such as cardiovascular disease, type 2 diabetes mellitus (T2DM), cancer, and neurocognitive disorder [4]. Walnuts, including their roots and leaves, have been traditionally used as herbal medicines for treating various conditions such as infectious disease, abdominal pain, skin disease and rheumatic pain [5,6]. Interestingly, in folk medicine, walnuts have also been used for treating liver injury, even though the efficacy remains uncertain [7].
Non-alcoholic fatty liver disease (NAFLD) is a disease whose prevalence is increasing in adults and children globally; its prevalence parallels that of obesity [8,9]. NAFLD is a broad spectrum disease whose manifestation range from simple steatosis and steatohepatitis to liver cirrhosis and liver cancer [10]. It is characterized by the excessive fat deposition in the hepatocytes, suggesting that NAFLD is a metabolic disease associated with dyslipidemia. However, NAFLD is not a simple metabolic liver disorder and is associated with various extra-hepatic diseases, including cardiovascular disease, chronic renal disease, and cancers as well as metabolic comorbidity with obesity, T2DM, and hyperlipidemia, which are interconnected as metabolic syndrome [11]. Although hepatic fat accumulation can be promoted by multiple metabolic and environmental factors, NAFLD is considered to be a nutritional disease. Lipid metabolism in the liver is a complex process involving digestion and absorption of foods and nutrients to obtain energy and maintain metabolic homeostasis.

Unfortunately, although NAFLD is the most common liver disease, no specific medications are available for NAFLD except those that target NAFLD-accompanying conditions such as dyslipidemia, obesity, high blood glucose or high blood pressure. Thus, lifestyle change such as diet control and exercise are recommendations for etiology-based therapy. In this respect, the management of NAFLD with walnuts or walnut-enriched diets can be an effective strategy because healthy components of walnut can target all candidate mechanisms underlying NAFLD development and progression.

The present review addressed the effects of walnut on NAFLD based on the pathophysiologic mechanisms by which walnut and walnut components exert preventive effects with respect to the development and progression of NAFLD.

2. Materials and Methods

In fact, publications regarding the relationship between walnut and NAFLD are few, even though clinical trials of walnut on the other health conditions—such as cardiovascular disease and T2DM—have been reported. To propose the usefulness of walnut, which has a variety of healthy components, for the prevention and treatment of NAFLD, we focused on the effect of walnut on the molecular and metabolic mechanisms underlying the NAFLD development and progression. We conducted the PubMed search to find publications from 2016 to 2020 to update recent knowledge and broaden our future perspectives. Nevertheless, to introduce the historical background or to give credit to the original observation we cited publications before 2016.

From a PubMed search with key words of NAFLD, NASH, liver cancer, and pathogenesis we found 387 papers have been published in the past 4 years. For the 3.1 Pathogenesis section, we focused on the contribution of hepatic cells on the NAFLD pathogenesis and 50 publications were found with keywords of NAFLD, pathogenesis, and liver cell. Individual intra- and extra-hepatic cells play the major roles in the physiologic functions of liver as well as the pathogenesis of NAFLD. To discuss about the influence of walnut on NAFLD pathogenesis at Section 3.2 we searched the PubMed with keywords of walnut and liver and found 98 publications including animal studies. We selected papers that may suggest how major components of walnut exert on the NAFLD pathogenesis individually or in combination. We tried to exclude the publications on nut in general rather than walnut specific papers because each type of nuts offers different nutritional benefits. Finally, we selected 100 publications by avoiding the overlap issue and choosing more recent ones.

For the better understanding of candidate mechanisms, we made three tables. Table 1 summarized the role of intra- and extra-hepatic cells in the pathophysiology of NAFLD, Table 2 summarized the individual walnut components that may mitigate the harmful effects of NAFLD, and Table 3 summarized animal and human clinical studies that evaluated the efficacy of walnut consumption on NAFLD.
3. Results and Discussion

3.1. Pathogenesis of NAFLD and Progression to NASH, Fibrosis, and Primary Liver Cancer

The liver performs a wide range of body functions essential for maintaining health. A key role of the liver is maintaining metabolic homeostasis by regulating the levels of macronutrients, such as glucose, lipid and protein [12]. Hepatocytes, which are major parenchymal cells in the liver, play pivotal roles in metabolism, detoxification, and protein synthesis. Hepatocytes can accumulate triglyceride by increasing fatty acid uptake and de novo lipogenesis, and metabolize fatty acids through lipolysis [13]. Kupffer cells, the resident macrophage in the liver, play an integral role in the hepatic immune response [14]. Hepatic stellate cells produce collagen and matrix when activated in response to liver injury and play a vital role in hepatic fibrogenesis [15] (Table 1).

In the early stage of simple steatosis, there is no or minimal damage to hepatocyte function. However, non-alcoholic steatohepatitis (NASH) causes hepatocyte damage that is represented by hepatocellular ballooning, formation of Mallory–Denk bodies, and lobular inflammation [16]. In the spectrum of NAFLD, liver injury results from a chronic inflammatory response by immune cells (Kupffer cells, neutrophils, dendritic cells, B cells, cytotoxic T cells and T-helper cells) and fibrosis by activated stellate cells [17,18].

3.1.1. Lipid Accumulation (Steatosis) and Lipotoxicity

Lipids are essential for maintaining cellular homeostasis, and therefore intracellular lipid accumulation by dysregulated lipid metabolism can cause lipotoxicity, which leads to organ dysfunction, cell injury, and cell death. Lipotoxicity associated chronic hepatic inflammation is known to trigger the development of NAFLD [19,20]. Fundamentally, excessive hepatic lipid accumulation results from an imbalance between triglycerides acquisition and disposal; acquisition occurs through increased release of free fatty acids from adipose tissue and intra-hepatic de novo lipogenesis, whereas disposal occurs through export of lipid via very low-density lipoprotein (VLDL) [21].

Aberrant lipid deposits alter the metabolic and inflammatory signals by modifying the biology and function of intracellular organelles such as the endoplasmic reticulum and mitochondria [22,23]. Other mechanisms include direct modification of intracellular signaling pathways and interaction with proinflammatory cellular kinases [24]. Toxic lipid species (triglycerides, free fatty acids, lysophosphatidyl choline, ceramides, and free cholesterol) induce mitochondrial dysfunction with oxidative stress through the production of reactive oxygen species (ROS) and endoplasmic reticulum stress, resulting in the activation of inflammatory signal and apoptosis [25–30].

3.1.2. Inflammation (Steatohepatitis) and Multiple-Hit Hypotheses

Previously, the development of NAFLD was described as a ‘two hits hypotheses’, consisting of hepatic lipid accumulation (steatosis) and consequent inflammation and fibrosis (steatohepatitis) [31]. Aberrant fat deposition in the hepatocytes may result from insulin resistance, followed by a sedentary lifestyle, a high-fat diet, and obesity. For the progression of simple steatosis to NASH, further inflammatory cascade and fibrogenesis signals are involved.

However, recent evidence suggested that the pathogenesis of NAFLD could be more complicated and multifactorial and proposed a ‘multiple-hit hypotheses’ rather than the two hits. As an etiologic mechanism underlying the development of NAFLD and progression to NASH, lipotoxicity has been proposed to be associated with insulin resistance, oxidative stress, and activation of inflammatory cascade. Hepatic lipotoxicity triggers and amplifies the inflammatory pathways by inducing the production of proinflammatory cytokines and activation of inflamasomes, which are multimeric protein complexes of the innate immune system responsible for the activation of inflammatory responses [32]. Inflammatory pathways, including c-Jun N-terminal kinase activator protein-1 (JNK-AP-1) and IκB kinase-nuclear factor kappa-light-chain-enhancer of activated B cells (IKK-NF-κB), are important for the maintenance of chronic inflammation during the development of
NAFLD [33]. The activation of inflammasomes, such as the Nod-like receptor proteins 3 (NLRP3), in response to free fatty acids, oxidative stress, and other proinflammatory metabolites, suppresses peroxisome proliferator-activated receptor-α (PPAR-α), and promotes tumor necrosis factor-α (TNF-α)-induced cell death [34]. Inflammasomes can directly promote fibrosis by activating hepatic stellate cells [35].

A disturbed intestinal microbiome is also implicated in the development and progression of NAFLD via the overproduction—and increased absorption—of fatty acids in the bowel [36,37]. Excessive free fatty acids activate inflammatory signaling pathways and release of proinflammatory cytokines such as interleukin (IL)-6, IL-1β and TNF-α to induce chronic hepatic inflammation [38]. Microbial products such as lipopolysaccharide also interact with receptors on inflammatory cells as well as with hepatic stellate cells to promote inflammation and fibrosis [38,39]. Along with steatosis and lipotoxicity, the regulation of cell death by apoptosis and autophagy is also defective in NAFLD [40–42].

3.1.3. Immune Reactions

Immune imbalance in the liver plays a pivotal role in the maintenance and progression of chronic inflammation in NAFLD. Recruitment and activation of immune cells induce the release of inflammatory mediators. In the liver, macrophages consist of unique tissue-resident macrophages, the so-called Kupffer cells, and bone marrow monocyte-derived macrophages. Although the primary function of Kupffer cells is to remove bacteria and microbial products from the portal system as the first immune cells in the liver, they also paradoxically contribute to the progression of simple steatosis into NASH via the production of TNF-α and C-C motif chemokine ligand 2 (CCL2) [14,43]. Kupffer cells express high levels of inflammasome components leading to the progression of NASH. The recruitment of bone marrow-derived monocytes is also important in the progression of NASH as they promote inflammation and fibrosis [44] (Table 1).

The role of liver dendritic cells in NAFLD remains unclear, but dendritic cells in conditions of increased intracellular lipid content exhibit a proinflammatory phenotype [45]. Neutrophils promote the progression of NASH through the release of cytokines, chemokines, active molecules, and neutrophil extracellular traps [46]. Neutrophil extracellular traps are composed of nucleic acids and antimicrobials and have been suggested to be important in chronic inflammatory conditions and in cancer progression. Markers of neutrophil extracellular traps are elevated in the serum of patients with NASH. T cell subsets also play differential roles in the progression of NASH. T-helper cells promote NASH through increased production of cytokines such as interferon-γ (IFN-γ) and IL-17, and decreased production of anti-inflammatory cytokines IL-4, IL-5, and IL-13 [47]. Cytotoxic T cells increase the production of IFN-γ and TNF-α, leading to inflammation in the liver [48,49]. The role of B cells in NAFLD is unclear, even though B cells have recently emerged as critical regulators of obesity-induced adipose tissue inflammation [50].

3.1.4. Insulin Resistance

Insulin resistance contributes to the dysfunction of adipose tissue and secretion of adipokines and inflammatory cytokines. Insulin resistance has been noted as one of key factors in the pathophysiology of NAFLD because this is frequently associated with obesity, T2DM, and metabolic syndrome, all of which can manifest as insulin resistance [10]. Insulin signaling in hepatocytes induces gluconeogenesis and plays an important role in regulating the fatty acid levels by inhibiting lipolysis as well as lipoprotein export. Development of insulin resistance in the adipose tissue increases peripheral lipolysis and free fatty acids can accumulate as triglycerides; the resulting hyperinsulinemia combined with diet can promote lipogenesis [51]. Progression of insulin resistance results in the release of non-esterified fatty acids by the adipose tissue into the bloodstream, resulting in postprandial lipidemia and fat deposition in the liver [52].

In recent years, the cause-and-effect relationship between insulin resistance and NAFLD has been reported to be bidirectional [53]. Systemic insulin resistance associ-
ated with central obesity and the metabolic syndrome is a major risk factor in NAFLD, while hepatic steatosis may aggravate metabolic disturbances through the hepatic insulin resistance, resulting in inactivation of insulin receptors [54].

3.1.5. Fibrosis and Hepatic Degeneration

Liver fibrosis is characterized by the excessive accumulation of the extracellular matrix, ultimately induces hepatic structural changes by a repetitive alternation of inflammation and anti-inflammatory response and immune repair, called fibrogenesis and fibrolysis [55,56]. When chronic inflammation persists, further metabolic deterioration affects liver fibrosis [57]. In liver fibrogenesis, activated hepatic stellate cells, and myelofibroblasts are the prime effector cells that exhibit proliferation, migration and contractility, and resistance to apoptosis. Among various extracellular matrix components, the synthesis and deposition of collagens are prominent in the liver cirrhosis.

In early liver disease, fibrogenesis is compensated by fibrolysis, which is characterized by the removal of excess extracellular matrix by proteolytic enzymes, mainly metalloproteinases. When fibrogenesis is more prominent than fibrolysis through an excessive synthesis and deposition of various extracellular matrix components, fibrolysis is compromised by increased synthesis of tissue inhibitor of metalloproteinases 1 (TIMP-1) and decreased production of fibrolytic metalloproteinases by hepatic stellate cells, myelofibroblasts, and Kupffer cells/macrophages. Other cells such as cholangiocytes, hepatic progenitor cells, endothelial cells, and other inflammatory cells responding to various stimuli can also contribute to fibrogenesis or fibrolysis.

3.2. Mechanisms by Which Walnut Contributes to the Management of NAFLD

3.2.1. Effects of Walnut on Metabolism

Lipid Metabolism

Walnut has a unique fatty acid composition; it is rich in linoleic acid as well as other compounds such as phytosterols, vegetable protein, and fiber, thereby having a positive effect on dyslipidemia (Table 2). A meta-analysis demonstrated that walnut-enriched diets can significantly reduce the blood levels of total cholesterol, low-density lipoprotein cholesterol, triglyceride, and apolipoprotein B compared with those of the control groups. More significant reductions in blood lipids were found upon comparing American and Western diet groups. Furthermore, adding walnut—which has 654.4 calories per 100 g—to diet did not induce body weight gain [58].

An early animal study using Zucker rats demonstrated that dietary lipids from walnut oil can reduce hepatic steatosis by decreasing the hepatic triglyceride level [59]. Another animal study using C57BL/6J mice demonstrated that walnut protected the liver from high-fat diet-induced hepatic triglyceride accumulation by modulating the levels of sirtuin 1 (Sirt1), 5′ adenosine monophosphate-activated protein kinase (AMPK), and FAS cell surface death receptor (FAS). This resulted in hepatic lipid homeostasis, suppression of adipose tissue inflammation and macrophage migration, and prevention of adipocyte apoptosis via a reduction in the levels of phosphorylated c-JNK (p-JNK) and phosphorylated p38 mitogen-activated protein kinases (p-p38K) [60].

A large prospective study combined with the Nurses’ Health Study and the Health Professionals Follow-up Study showed that individuals consuming one serving of nuts at least five times per week had a 14% lower risk of cardiovascular disease and a 20% lower coronary artery disease compared with those of individuals with lowest nut consumption. Interestingly, walnut consumption (one or more times/week) was more strongly associated with a lower risk of total cardiovascular disease and coronary artery disease compared with the risk associated with consumption of other nuts [61].

Evidence also indicated that walnuts are also useful for the prevention of T2DM. A Nurses’ Health Study revealed a protective effect of walnut consumption on the development of T2DM [62]. The National Health and Nutrition Examination Survey data demonstrated that walnut consumers were at a lower risk of developing T2DM than
non-nut consumers and also had lower levels of fasting blood glucose and hemoglobin A1c (HbA1c), which reflects the average blood glucose levels for the last two to three months [63]. In a randomized, double-blind, placebo-controlled clinical trial, the addition of walnut oil into the daily diet of T2DM patients improved the lipid profiles. Consumption of walnut oil significantly decreased the blood levels of total cholesterol, low-density lipoprotein, and triglyceride compared with their levels in the control group [64]. Collectively, walnut helps maintaining healthy cholesterol and triglyceride levels. Currently, there is no direct evidence from human studies regarding the effect of walnut on the lipid profile of NAFLD patients, and only animal study data are available (Table 3). However, walnut, which can improve the lipid profile—in human—and reduce the risk of cardiovascular disease and correct dyslipidemia in diabetes mellitus, may provide beneficial effects for NAFLD patients by correcting dyslipidemia and thereby reducing steatosis.

One-Carbon Metabolism

One-carbon metabolism is interrelated metabolic pathways that produce purines and thymidylylate through the nucleotide synthesis pathway, S-adenosylmethionine through the transmethylation pathway and remethylation pathway, and glutathione and taurine through the transsulfuration pathway. One-carbon metabolism also metabolizes toxic homocysteine through remethylation pathway and transsulfuration pathway. Folate, vitamin B-12, vitamin B-6, and vitamin B-2 are coenzymes of one-carbon metabolism and methionine, choline, betaine, serine, glycine, threonine and glucose are the methyl donors for this metabolism [65]. The liver is the major site of one-carbon metabolism and produces most of the S-adenosylmethionine, which is the unique methyl donor for many biological methylation reactions and is involved in VLDL secretion [66]. VLDL is a triglyceride-rich lipoprotein, and impaired excretion of VLDL from the liver may result in excessive fat accumulation in the liver [67].

Previous animal studies suggested that methyl-deficient diets (deficient in folate, vitamin B-12, methionine, and choline, all of which are one-carbon nutrients) can induce fatty liver changes and increase hepatic cell turnover, ultimately leading to the development of hepatic tumors in the animal model. A plausible mechanism is that the deficiency of one-carbon nutrients may lead to reduced VLDL secretion and fat accumulation in the liver [66]. Moreover, methyl deficiency induces low S-adenosylmethionine pools, thereby resulting in DNA hypomethylation. This, in turn, leads to changes in the expression of genes that may play key roles in the regulation of growth and cell cycles, ultimately resulting in the development of hepatic tumor. As methyl-deficient diet can induce fatty liver, folate, vitamin B-12, methionine, and choline were called lipotropes, which mean compounds that prevent excess fat deposition.

Methionine adenosyltransferase catalyzes the synthesis of S-adenosylmethionine from methionine in the liver. In a methionine adenosyltransferase deficient mouse model, the low hepatic S-adenosylmethionine level resulted in reduced phosphatidylcholine and polyunsaturated fatty acids levels as well as impaired synthesis and release of VLDL-triglyceride, leading to the accumulation of triglyceride, diacylglycerol, and fatty acids. Accumulation of oxidized fatty acids in the liver, subsequent oxidative stress, and abnormal hepatic lipid status trigger the spontaneous development of steatosis and the progression to NASH and fibrosis [68], suggesting that S-adenosylmethionine deficiency may be a critical driver of NAFLD. S-adenosylmethionine supplementation in this mouse model corrected many of the biochemical abnormalities and resulted in a return to near normal liver histology and triglyceride levels. S-adenosylmethionine supplementation in rats fed a lipogenic methionine- and choline-deficient diet also improved liver histology. Fortunately, except for vitamin B-12, walnut is rich in all sorts of one-carbon nutrients, including folate, vitamin B-6, methionine, serine, glycine, threonine, glucose, choline, and betaine, all of which involve one-carbon metabolism, derangement of which can induce fatty liver.

Accumulating evidence suggests that certain bioactive components from plant foods might exhibit beneficial effects on health through the modification of epigenetic phenomena.
that affect gene expression without altering DNA base pairs. The effects of dietary polyphenols appear to be manifested either through the direct inhibition of DNA methyltransferases or indirectly through metabolic effects associated with energy metabolism. Nutrients and dietary bioactive food components can alter gene expression through alterations in DNA methylation, histone modification, and microRNA; therefore, an “epigenetic diet” has been proposed, which leads to beneficial health outcomes through the modification of the epigenome. Walnut contains a plenty of one-carbon nutrients, polyphenols, and other bioactive food components and could therefore be used as a component of epigenetic diet that affects NAFLD through epigenetic mechanism. Further studies are warranted to clarify the epigenetic effects of walnut on NAFLD.

Insulin Resistance and Glucose Metabolism

Insulin resistance, which is closely associated with T2DM and obesity, is a manifestation of NAFLD, and individuals with NAFLD is known to exhibit impaired hepatic glucose production similar to patients with overt T2DM. Thus, NAFLD is independently associated with increased levels of fasting blood glucose and HbA1c.

In a randomized clinical trial in 20 patients with T2DM, supplementation with α-linoleic acid, which is rich in walnut, improved glucose homeostasis and insulin sensitivity with an increase in the level of adiponectin that is known to decrease hepatic and systemic insulin resistance and attenuate hepatic inflammation and fibrosis. In a crossover randomized clinical trial conducted with ten obese subjects, walnut consumption significantly decreased insulin resistance along with a reduction in the blood levels of ceramides and sphingomyelins that are known to promote insulin resistance. A randomized controlled clinical trial in 100 patients with T2DM demonstrated that consumption of walnut oil (15 g/day for 3 months) significantly reduced HbA1c and fasting glucose levels with respect to the baseline. Collectively, these studies indicate that walnut may also exert beneficial effects on NAFLD-associated insulin resistance and impaired glucose metabolism.

3.2.2. Effects of Walnut on Lipotoxicity (Oxidative Stress)

Lipotoxicity refers to cellular damage caused by the aberrant accumulation of lipid compounds in the cell, resulting in oxidative damage to tissue and organs through the formation of ROS. In NAFLD patients, abnormal lipid metabolism causes damage to mitochondrial DNA and mitochondrial respiratory chain proteins, thereby increasing ROS formation. This phenomenon is particularly important for the progression of NAFLD to NASH. In NASH, the levels of toxic palmitate are elevated in the plasma and palmitate accumulates is observed in the liver, where it can activate apoptosis process in hepatocytes.

Walnut has a very high content of antioxidants. A randomized crossover study demonstrated that walnut meal increased postprandial plasma concentrations of γ-tocopherol, galloctechn gallate, epicatechin gallate and epicallocatechin gallate and increased hydrophilic and lipophilic oxygen radical absorbance capacity in healthy individuals.

In a high-fat diet animal model with C57BL/6J mice, walnut supplementation not only decreased hepatic fat accumulation but also significantly decreased the hepatic levels of cytochrome P450-2E1, an important source of oxidative stress, nitrated proteins—which are markers of nitrosative stress and are involved in the development and progression of NAFLD—and lipid peroxidation, a metabolic process wherein ROS result in the oxidative deterioration of lipids. Furthermore, walnut supplementation decreased the levels of activated cell death-associated p-JNK and p-p38K, a phenomenon that was accompanied by increased hepatocyte apoptosis in the high-fat-diet group. These results collectively suggested the beneficial effects of dietary walnut, i.e., attenuating high-fat diet-induced hepatic steatosis and apoptosis could be partially attributed to the anti-oxidant ingredients.

In a transgenic mouse model of Alzheimer’s disease, transgenic mice fed a control diet showed significant age-dependent increase in ROS levels, lipid peroxidation, and protein oxidation, coupled with impaired activities of anti-oxidant enzymes such as super-
oxide dismutase, catalase, and glutathione peroxidase compared with those in wild-type mice. Transgenic mice fed diets containing walnuts exhibited significantly reduced oxidative stress, as demonstrated by decreased levels of ROS, lipid peroxidation, and protein oxidation and enhanced activities of anti-oxidant enzymes compared with those in the control diet mice. Long-term supplementation with walnuts was more effective in reducing oxidative stress in this animal model [78]. These observations indicate that walnut can reduce oxidative stress, not only by scavenging free radicals, but also by maintaining the anti-oxidant status. Walnut has potent anti-oxidant properties and may reduce the oxidative stress associated with lipid accumulation in NAFLD.

3.2.3. Effects of Walnut on Inflammation and Immunity

Inflammation

Walnut polyphenols have been studied for treating inflammation [5]. In addition, walnut is exceptionally rich in α-linolenic acid, a plant-based omega-3 polyunsaturated fatty acids, and fibers in walnut also have anti-inflammatory properties. Walnut is also an excellent source of γ-tocopherol, which is a potent free radical scavenger and reduces proinflammatory eicosanoids and inflammatory responses.

In a colon cancer cell line, COLO205, walnut phenolic extracts significantly inhibited IL-8 and IL-1α expression and attenuated both TNF-α-induced inhibitor of nuclear factor kappa B (IkB) phosphorylation/degradation and the DNA binding activity of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). The oral administration of walnut phenolic extracts significantly reduced the severity of colitis in both acute and chronic colitis models [79]. In a systemic review and meta-analysis of randomized controlled trials, nut consumption improved vascular endothelial function but not C-reactive protein, the most common inflammatory maker for the evaluation of anti-inflammatory effects of food or diet [80], whereas a two-year supplementation of walnut to the diet improved the expression of six inflammatory markers, i.e., granulocyte-macrophage colony-stimulating factor (GM-CSF), INF-γ, IL-1β, IL-6, TNF-α, and sE-selectin, suggesting the long-term effects of walnut on meta-inflammation, a chronic sustained subclinical inflammation associated with obesity, diabetes mellitus and aging [81].

Collectively walnut—with its abundant anti-inflammatory components—may be effective against inflammation associated with NAFLD.

Immunity

Recent evidence suggests that immunologic imbalances in the liver drive the development and progression of NAFLD. In NASH, immune cells are recruited into the liver and activation of these cells releases inflammatory molecules. Although the innate immune mechanism is considered a major contributing factor, recent evidence indicates that adaptive immunity also plays an important role in the progression of NAFLD to NASH [70]. Walnut contains many immune-potentiating components such as vitamin E, vitamin B-6, copper, zinc, and selenium. Recent studies have also suggested that walnut hydrolyzates, walnut polyphenolic extracts, and walnut oligopeptide are effective immune boosters in several animal studies.

In an animal study using BALB/c mice, walnut protein hydrolyzates (albumin, glutelin, and globin) improved immune indices such as splenic lymphocyte proliferation, phagocytic activity of macrophage, number of CD4+ and CD8+ T cells, and IgA, suggesting the use of walnut proteins as nutritional resources to boost the immune function [82].

In another animal study that employed specific pathogen-free Kunming mice [83], walnut phenolic extracts significantly attenuated immunotoxicity in splenocytes exposed to 4-pentylphenol and 3-methyl-4-nitrophenol, both of which are components of vehicle emissions that are known to be toxic to spleen, an immune organ that links innate and adaptive immunity [84]. Treatment with walnut phenolic extracts—"containing least 16" phenols, including ellagitannins, quercetin, and gallic acid—was shown to significantly increases in the percentages of splenic T lymphocytes (CD3+ T cells) and T cell subsets
(CD4+ and CD8+ T cells), as well as the production of T cell-associated cytokines (IL-2 and IL-4,) in cells exposed to the splenotoxins.

In a recent animal study using BALB/c mice [85], walnut oligopeptide significantly improved humoral- and cell-mediated immune responses, macrophage phagocytosis, and natural killer cell activity, along with T-helper cell stimulation and increased the production of cytokines and immunoglobulin. Food-derived peptides have been suggested as effective and safe immunomodulatory supplements [86], as demonstrated by their positive effects on macrophages. The bioavailability of peptides is strongly associated with specific amino acid sequences and amino acid composition; therefore, the high protein levels and balanced amino acid composition in walnut contribute to its immune-potentiating activity.

Walnut has both anti-inflammatory and immune-enhancing components, and walnut consumption may prevent the development and progression of NAFLD.

3.2.4. Effects of Walnut on Stemness

Hepatic stem/progenitor cells are bipotent progenitor cells that can differentiate into either hepatocytes or cholangiocytes. They are quiescent during physiologic conditions but are activated in response to acute and chronic liver injuries and play a critical role in regeneration and repair. Hepatic stem/progenitor cell activation appears to be a physiologic response of the liver to oxidative stress in NAFLD [75].

Even though there is no direct evidence regarding the effects of walnut on hepatic stem/progenitor cells, the results of two animal studies indicate a beneficial effect. In an Adenomatous Polyposis Coli (APC)-deficient animal model of colon cancer, walnuts seem to preserve intestinal stem cell function in high-fat diet conditions [87]. To assess the intestinal stem cell function, crypts were isolated from the small intestine of mice fed either a low-fat, high-fat, or high-fat diet plus walnut for four weeks. An ex vivo 3D intestinal organoid assay revealed that four weeks of a high-fat diet significantly reduced the ability of intestinal stem cells to generate organoid-like structures, but walnut consumption reversed the high-fat diet-induced decline in intestinal stem cell function. Consumption of walnut—containing a high concentration of α-linolenic acid—was associated with increased hepatic docosahexanoic acid levels in mice; this compound has been shown to induce neuronal differentiation of gliogenic neural stem/progenitor cells [88]. Further studies are needed to determine the effects of walnut or walnut components on hepatic stem/progenitor cells to enable the use of walnut for hepatic regeneration from the hepatic cell death and fibrosis in NAFLD.

3.2.5. Walnut and Gut Microbiota

Fiber and polyphenols—abundant in walnuts—exert a prebiotic effect and alter the gut microbiota profile, suggesting that walnuts may correct dysbiosis associated with NAFLD. An ability to influence gut microbiota may also be useful for the management of NAFLD because gut microbiota are known to influence the lipid metabolism, glucose metabolism, insulin resistance, immune function, and mucosal and systemic inflammation.

In a meta-analysis of randomized controlled trials that determined the metabolic effects of microbiome-targeted therapies, the use of probiotics/synbiotics was associated with improvement in the levels of liver-specific markers of hepatic inflammation, liver stiffness, and steatosis. This study suggested that modulation of gut microbiota can be useful for the management of NAFLD [89].

The effects of walnut on gut microbiota were also examined in a controlled-feeding, randomized crossover study in healthy men and women who received isocaloric diets containing either 0 or 42 g walnuts per day for three weeks, with a one-week washout period between the two diet periods. Walnut consumption affected the gastrointestinal microbiota profile with higher relative abundance of Faecalibacterium and Clostridium and lower relative abundances of Ruminococcus and Bifidobacterium, and reduced LDL-cholesterol. These results suggest that walnuts may provide health benefits through the modulation of gut microbiota [90].
In a randomized, controlled, prospective, crossover study [91], a walnut-enriched diet was provided to the subjects, and 16S rRNA gene sequencing was conducted using the stool samples. Daily intake of 43 g walnuts for eight weeks significantly affected the gut microbial environment by enhancing probiotic- and butyric acid-producing species in healthy individuals.

An animal study using Fischer 344 rats also demonstrated that alterations in the gut microbial profile occurred after consuming a diet containing walnut [92]. Dietary walnuts increased the abundance of Firmicutes and reduced the abundance of Bacteroidetes. Walnuts increased the abundance of probiotic-type bacteria including Lactobacillus, Ruminococcaceae, and Roseburia but decreased that of Bacteroides and Anaerotruncus. Altering the gut microbial environment might be a new mechanism by which walnuts confer their beneficial health effects.

Clinical trials and animal study have suggested that walnut, which is rich in fiber, polyphenol, polysaccharides and α-linolenic acid, exert positive effects on gut microbiota signatures, resulting in a positive effect on the gut health. Thus, walnuts might affect NAFLD by modulating the gut microbiota.

4. Conclusions and Future Perspectives
Walnut is one of the most popular nuts globally. It is a common dietary ingredient that is an important component of the Mediterranean diet and Asian diets. Over the past few decades, the health effects of walnut consumption have been extensively investigated as walnut contains a variety of nutrients and bioactive components. Due to the global increase in obesity, hyperlipidemia, T2DM, and metabolic syndrome, NAFLD has attracted more attention because it is a hepatic manifestation of these unhealthy conditions. Furthermore, because NAFLD can progress to NASH and subsequently result in the development of hepatic fibrosis and hepatocellular carcinoma, the incidence of NAFLD-associated hepatocellular carcinoma is expected to increase, whereas the incidence of hepatitis B and C viruses associate hepatocellular carcinoma is declining after the introduction of antiviral agents effective against these two viruses.

However, because there is no specific medicine available for NAFLD, metabolic correction with nutritional intervention could be a major strategy to enable treatment for this condition. Over recent decades, accumulating evidence from a series of clinical and experimental studies has demonstrated that walnut phenolic and lipid extracts, walnut hydrolysate, walnut oil and walnut-enriched diet have consistently demonstrated the health effects on aberrant lipid and/or glucose metabolisms as well as metabolic disorders including NAFLD. We have described here that consumption of walnuts, which are rich in a range of nutrients and bioactive components, can ameliorate aspects of NAFLD pathophysiology such as steatosis, lipotoxicity, steatohepatitis, insulin resistance, down-regulated immunity and gut microbiota dysbiosis, thereby alleviating NAFLD and hopefully preventing its progression towards NASH and fibrosis.

Future studies should focus on the molecular mechanisms by which walnuts exert protective effect against NAFLD development, progression to NASH and fibrosis, and ultimately hepatocarcinogenesis. More evidence is needed to understand the effects of walnut on hepatic stem cells and on the gut microbiota. The effects of walnut on alcoholic liver disease, which is similar in some respects to NAFLD, will also be an interesting subject. NAFLD development is also associated with genetic makeup such as the Patatin-like phospholipase domain-containing 3 (PNPLA3) polymorphism [93]; therefore, investigating the interactions between walnut and genes would be helpful to understand the individually different effects of walnut on NAFLD.

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Table 1. Pathophysiologic mechanisms of NAFLD focused on the functions of individual hepatic cells.

| Cell Type                          | Cell Oriented NAFLD Pathophysiology                                                                 |
|-----------------------------------|-----------------------------------------------------------------------------------------------------|
| Intra-hepatic cells               |                                                                                                      |
| Hepatocyte                        | Triglyceride storage (steatosis), lipotoxicity, oxidative stress, nitrosative stress, mitochondrial dysfunction, endoplasmic reticulum stress, apoptosis, insulin resistance |
| Kupffer cell (resident liver macrophage) | Inflammation (steatohepatitis), production of inflammatory chemokines and cytokines                     |
| Hepatic stellate cell             | Production of extracellular matrices as well as proinflammatory and profibrogenic cytokines to produce fibrosis |
| Myelofibroblast (derived from hepatic stellate cell and portal mesenchymal cells) | Main effector of fibrosis and wound healing response including regeneration and angiogenesis            |
| Cholangiocyte                     | Cholestasis and biliary ductular reaction as well as proinflammatory status associated with cholangiocyte senescence |
| Hepatic stem/progenitor cells     | Regeneration and repair after activated in response to oxidative stress                                |
| Macrophage (derived from bone marrow monocyte) | Infiltrate into the liver in response to hepatic metabolic or toxic damage and contribute to both the progression and resolution of tissue inflammation |
| Extra-hepatic cells               |                                                                                                      |
| Leukocyte                         | Recruited in hepatocyte injury, and activate inflammasomes and produce inflammatory cytokines and chemokines |
| Dendritic cell, natural killer cell, T cell subsets | Immune response and inflammation                                                                     |

Table 2. Walnut constituents that target the NAFLD-associated pathophysiologic mechanisms.

| NAFLD-Associated Mechanisms          | Walnut Constituents                                                                 |
|-------------------------------------|-------------------------------------------------------------------------------------|
| Lipid metabolism                    | Essential fatty acids (α-linolenic acid and linoleic acid), phytosterols, vegetable protein, fiber |
| One-carbon metabolism               | Folate, vitamin B-6, methionine, serine, glycine, glucose, choline                   |
| Insulin resistance and glucose metabolism | Walnut oil, fiber, magnesium                                                           |
| Oxidative stress with lipotoxicity  | Polyphenols, γ-tocopherol, melatonin, selenium                                         |
| Inflammation                        | Walnut phenolic extracts, fiber, α-linolenic acid, γ-tocopherol                         |
| Immune function                     | Walnut oligopeptide, walnut protein hydrolysate, walnut phenolic extract, γ-tocopherol, vitamin B-6, copper, zinc, selenium |
| Stemness                            | Phenolic compounds, walnut lipid extracts, walnut oil                                |
| Gut microbiota                      | Fiber, polyphenol, polysaccharide, α-linolenic acid                                  |
Table 3. Animal and human intervention studies explored the effects of walnut on NAFLD and NAFLD-associated metabolic conditions.

| Subjects                  | Type and Amount of Walnut                                                                 | Study Results Suggesting the Role of Walnut in NAFLD                                                                 | Ref  |
|---------------------------|-------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------|------|
| Female Zucker rat         | Walnut oil (8% or 14% added to the AIN93G diet)                                            | Inhibited hepatic lipid accumulation                                                                                | [59] |
| Male C57BL/6J mice        | Walnut (21.5% of energy) added to a high-fat diet (45% of total energy as fat)            | Reduced hepatic triglyceride amount and modulated the levels of proteins that involve in the hepatic lipid homeostasis such as Sirt 1, AMPK, FAS, and PPAR-α | [60] |
| Male C57BL/6J mice        | Walnut (21.5% of energy) added to a high-fat diet (45% of total energy as fat)            | Attenuated hepatic steatosis and apoptosis induced by the high-fat diet                                             | [77] |
| Male ddY mice             | Walnut phenolic extract (50–200 mg/kg with 45% phenol) added to high-fat diet (32% of total energy) | Reduced the liver weight and triglyceride level through enhancing peroxisomal β-oxidation                         | [94] |
| Male Wistar-albino rats   | Walnut oil (20 mg/kg) added to a high carbohydrate diet (20% of sucrose in drinking water) and a high-fat diet (42% of total energy) | Reduced the hepatic levels of MDA, a marker for oxidative stress, and NF-κB, as well as increased the level of e-NOS | [95] |
| Male Chinese Kung Ming mice | Walnut seed coat (100 mg/kg) via oral gavage with D-galactose injection                  | Alleviated the hepatocyte apoptosis, necrosis and inflammatory cell infiltration                                      | [96] |
| Male C57BL/6J mice        | Walnut oil capsule (6, 12, 18 mL/kg) through intragastric administration with D-galactose injection | Improved hepatic necrosis, hydropic degeneration, vacuolar degeneration, and lymphocytic infiltration along with increased total anti-oxidant capacity | [97] |
| Male Wistar rats          | Walnut-enriched diet (2.4 g/day) with high fructose in drinking water (10% w/v)          | Lowered the n-6/n-3 ratio in plasma, liver and epididymal adipose tissue, and increased LA and αLNA contents in the liver | [98] |
| Participants with obesity (n = 10) | Randomized placebo-controlled crossover clinical trial with five-day consumption of either walnuts (48 g/day) or placebo | Improved the lipid profile and reduced insulin resistance and blood levels of ceramides, sphingomyelins, and N-glycans | [74] |
| Participants with abdominal obesity and dyslipidemia (n = 278) | Eighteen-month randomized controlled trial with Mediterranean and low carbohydrate diet enriched with walnut at 28 g/day versus low-fat diet | Reduced the hepatic fat content compared with a low-fat diet                                                        | [99] |
| Patients with T2DM (n = 100) | Randomized controlled clinical trial with a walnut oil (15 g/day) added to their diet for 3 months | Improved the levels of fasting blood glucose and hemoglobin A1c                                                   | [64] |
| Health subjects (n = 40)  | Randomized controlled crossover study with a walnut-enriched diet (43 g/day) for eight weeks | Reduced the plasma levels of non-HDL-cholesterol and apolipoprotein B levels                                       | [100] |

sirtuin 1 (Sirt1), AMP-activated protein kinase (AMPK), FAS cell surface death receptor (FAS), peroxisome proliferator-activated receptor-α (PPAR-α), malondialdehyde (MDA), nuclear factor kappa B (NF-κB), endothelial-NO synthase (e-NOS), linoleic acid (LA), α-linolenic acid (αLNA), Deutschland, Denken, and Yoken (ddY), type 2 diabetes mellitus (T2DM).
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