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Role of Nrf2 and oxidative stress in the progression of non-alcoholic steatohepatitis to hepatocarcinoma

Papel de Nrf2 y estrés oxidativo en la progresión de esteatohepatitis no alcohólica a hepatocarcinoma

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ABSTRACT

Oxidative stress and oxidation-reduction reactions are present in all biological processes. In patients with metabolic syndrome, they contribute to the development of non-alcoholic steatohepatitis, cirrhosis, and hepatocellular carcinoma, through processes of lipotoxicity, cytokine recruitment, stellate cell activation, progressive collagen synthesis, DAMPs (injury-associated molecular pattern), PAMPs (pathogen-associated molecular pattern) and irreversible damage to mitochondrial DNA. Nrf2 is the most important transcription factor in the regulation of enzymatic antioxidant response gene expression and plays a major role in the cytoprotective response to acute inflammation and liver regeneration. However, it has also been associated with the development of chemoresistance and tumor recurrence. In this article, we review the existing evidence in this regard.

RESUMEN

El estrés oxidativo y las reacciones de óxido-reducción se encuentran presentes en todos los procesos biológicos. En pacientes con síndrome metabólico, contribuyen al desarrollo de esteatohepatitis no alcohólica, cirrosis y carcinoma hepatocelular, mediante procesos de lipotoxicidad, reclutamiento de citocinas, activación de células estelares, síntesis progresiva de colágena, DAMPs (patrón molecular asociado a lesiones), PAMPs (patrón molecular asociado a patógenos) y daño irreversible al ADN mitocondrial. Nrf2 es el factor de transcripción más importante en la regulación de la expresión de los genes de la respuesta antioxidante enzimática y juega un papel primordial en la respuesta citoprotectora ante la inflamación aguda y en la regeneración hepática. Sin embargo, se ha asociado también al desarrollo de quimiorresistencia y recurrencia tumoral. En este artículo, hacemos una revisión sobre la evidencia existente al respecto.

OXIDATION AND REDUCTION REACTIONS AND THEIR RELATIONSHIP TO LIVER DISEASE

Oxidation and reduction (redox) reactions are present in almost every biological process. The interactions between endogenous and exogenous oxidants and antioxidant systems, both enzymatic and non-enzymatic, have fundamental pathophysiological implications, from acid-base balance, bioenergetic processes, oxidation of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA), as well as their mutations, to a state of excessive oxidation related to the development of diseases. In aerobic metabolism, these reactions produce free radicals. Free radicals are unstable chemical molecules that have an unpaired electron and are characterized by being highly reactive with other molecules and are by-products of fatty acid metabolism and oxidation in peroxisomes, mitochondria, and smooth endoplasmic reticulum. The most common free radicals are superoxide anion (O_2^-), hydroxyl radical (OH^-), peroxyl radical (ROO^-), and alkoxy radical (RO^-), of which the hydroxyl radical is

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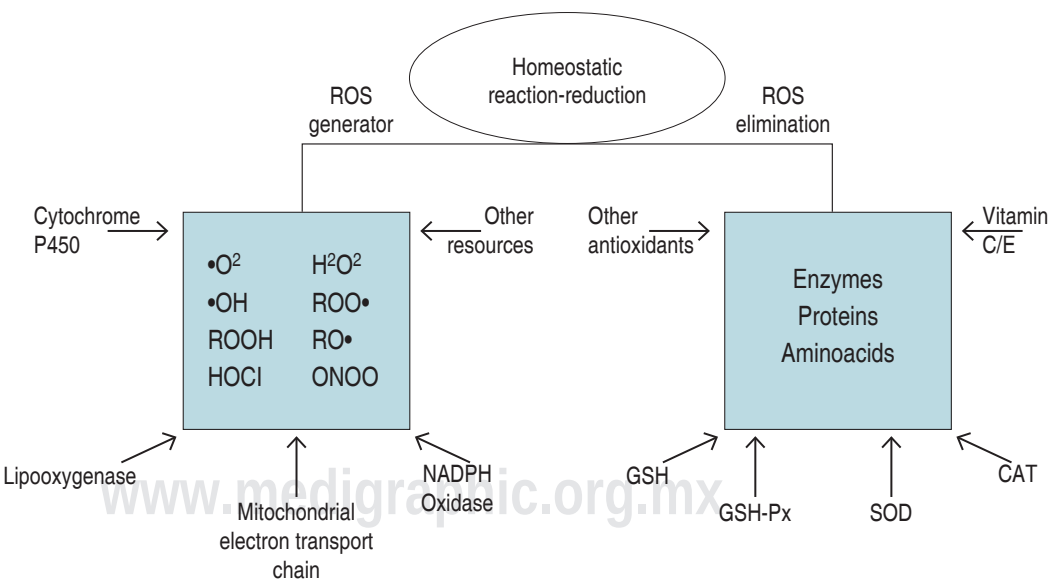
the most potent, subtracting a hydrogen atom from the methylene group (-CH_2^-) of fatty acids, leaving a free bond on the carbon atom (-CH^-), initiating a chain reaction, taking hydrogenions from the other fatty acid molecules until the substrate is totally consumed or the reaction is stopped by an antioxidant molecule. There are also other free radicals not associated with oxygen[1,2] *(Figure 1)*.

Oxidative stress is defined as the imbalance between the production of oxidative molecules and the synthesis of antioxidant molecules, leading to tissue damage.[3,4] In patients with non-alcoholic steatohepatitis (NASH), oxidative stress causes hepatocyte dysfunction or death, affecting enzymes involved in lipid metabolism.[5] Kupffer cells and neutrophils are mainly responsible for the hepatic production of free radicals.[6]

The antioxidant response is regulated by the nuclear factor κβ (NFκβ), the transcription factor AP-1, and the nuclear factor erythroid or respiration factor-associated factor (Nrfs) family of factors, of which Nrf2 is the most important regulator of gene expression involved in the enzymatic antioxidant system. Under normal conditions, Nrf2 is found in the cytoplasm attached to KEAP1, a cytosolic anchoring protein. But during increased oxidative stress Nrf2 detaches from KEAP1 and is translocated to the nucleus, where it activates gene transcription of antioxidant systems, increasing the expression of superoxide dismutase, catalase, glutathione peroxidase 2, glutathione reductase, the thioredoxin, myeloperoxidase, gamma-glutamylcysteine synthetase, and heme oxygenase-1 systems. Hepatocellular Nrf2 activation plays an important role in the cytoprotective response of the liver to oxidative stress. Multiple studies show that hepatocellular Nrf2 concentration is elevated during chronic and persistent oxidative stress processes, acute inflammation, and liver regeneration.[7,8]

PROINFLAMMATORY STATES AND LIPOTOXICITY-INDUCED FIBROGENESIS IN NON-ALCOHOLIC STEATOHEPATITIS

Non-alcoholic steatohepatitis is the most frequent liver disease in the world and there



Adapted from: Li S, et al.[30]

Figure 1: *The hemostatic reaction-reduction in the liver.*

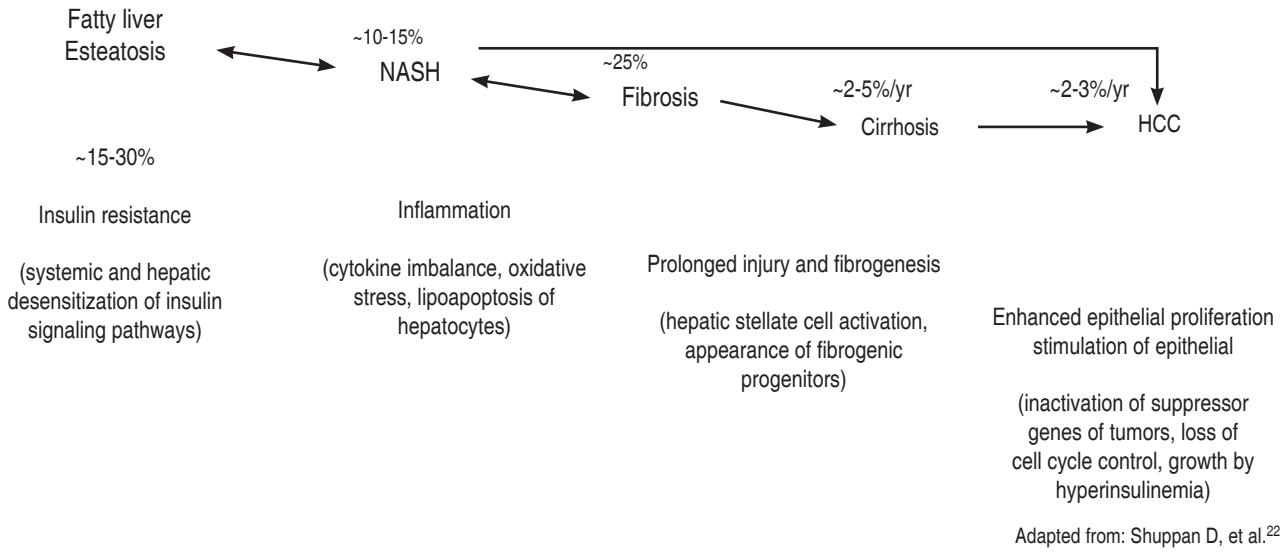
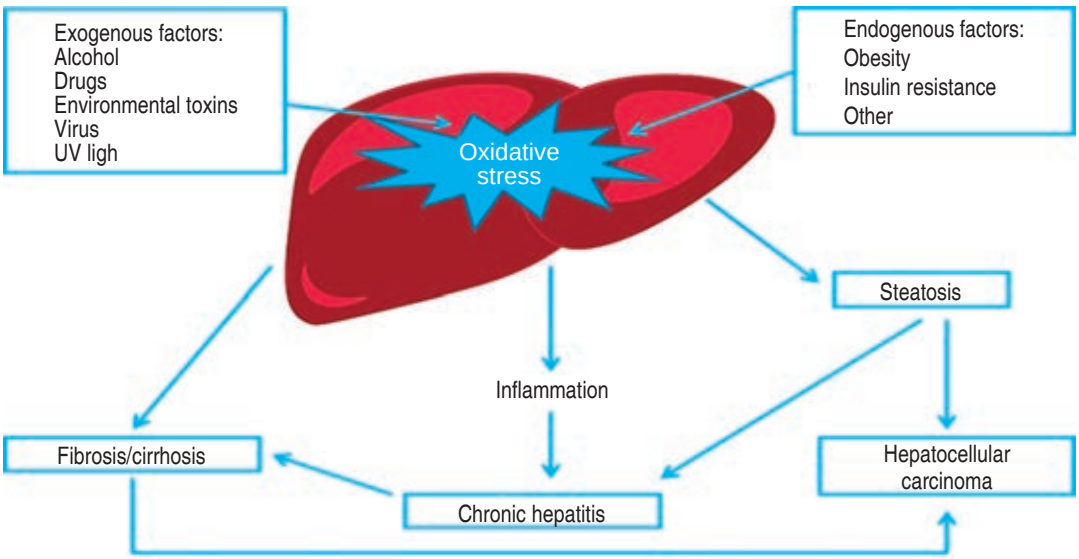


Figure 2: *Spectrum of non-alcoholic fatty liver disease. Estimated risks of progression to hepatocellular carcinoma and non-alcoholic steatohepatitis.*
HCC = hepatocellular carcinoma. NASH = non-alcoholic steatohepatitis.

is currently a trend in the increase of liver transplantation for cirrhosis.[9] It increases the spectrum of damage by converting simple hepatic steatosis (NAFL), without evidence of inflammation, into a pattern of progressive fibrosis called steatohepatitis (NASH). It is directly associated with insulin resistance, obesity, diabetes, and dyslipidemia, and is considered the main hepatic manifestation of the metabolic syndrome.[10,11] It has an overall prevalence of 59.1%[12] by diagnosis through liver biopsy, which increases to 95% in morbidly obese patients.[13-15] The disease is asymptomatic and slowly progressive, with progression between each degree of fibrosis for every 7.1 years in patients with steatohepatitis and 14.3 in patients with simple steatosis, of which 23% progress to fibrosis and cirrhosis,[16] and up to 7.6% may develop hepatocellular carcinoma[17] *(Figure 2)*. In the Americas, there is a direct correlation between obesity and non-alcoholic fatty liver disease (NAFLD), with a prevalence of 80% in obese patients.[18] A cross-sectional study conducted in 2012 found a prevalence rate of 84.1% of NAFLD diagnosed by ultrasound in patients younger than 60 years with metabolic syndrome[19] *(Figure 3)*.

Non-alcoholic steatohepatitis is the result of the sum of events and risk factors that begin with the increase in the concentration of free fatty acids in the portal circulation due to the overnutrition of patients with obesity, which produces an increase in triglycerides in hepatocytes and gluconeogenesis. The expansion of adipose tissue increases the production of adipokines, and the synthesis of proinflammatory cytokines such as monocyte chemotactic protein 1 (MCP1), IL-6, and IL-8, decreases the levels of adiponectin, a counter-regulatory adipokine stimulated by lipid accumulation in adipose tissue and liver, also increases circulating leptin concentrations, promoting fibrogenesis by stimulation of stellate cells. On the other hand, Kupffer cells produce proIL-1β, IL-12, and TNF-α, which add to the release of lipopolysaccharides (LPS) from the intestinal microbiome and inflammasomes, which are defined as protein complexes that act as sensors and mediators of inflammation related to specific sequence patterns of different pathogens or damage patterns, such as PAMPs and DAMPs. These activate Toll-like receptors-4 (TLR4) in the liver, increasing the synthesis of caspase 1 (Cas1) and thus inducing the maturation of proinflammatory cytokines IL-1β and IL-8. The production of proinflammatory factors by platelets and Kupffer cells stimulates stellate cells, or Ito cells, in the space of Disse



Adapted from Li S, et al.[30]

Figure 3: *Schematic of general mechanisms of oxidative stress induced by multiple factors in liver disease.*

to proliferate and transform into myofibroblasts. Subsequently, the "activated" stellate cells self-stimulate to increase production of TGF-β and TNF-α, paracrine activating adjacent stellate cells, perpetuating fibrogenesis, even when the initial damage has ceased. The result is persistent inflammation, fibrosis, and apoptosis of hepatocytes, mediated by an interrelated process of lipotoxicity, cytokine recruitment, mitochondrial dysfunction, and oxidative stress[20] *(Figure 4)*.

OXIDATIVE STRESS AND DNA DAMAGE IN NASH

Oxidative stress also produces epigenetic alterations to mitochondrial and nuclear DNA. Under normal conditions, oxidative damage to nucleosides maintains a balance between DNA oxidation and DNA repair. But under conditions of high oxidative stress, the damage is irreversible, and the tissue concentration of damaged DNA increases. This imbalance has a positive association with the aging process, carcinogenesis, and NASH. The main product of DNA damage due to oxidative stress is 8-hydroxy-deoxy-deoxy-guanosine (8-OHdG), which has been described as an early biomarker of tissue damage. Takahashi et al.[21] examined, through immunohistochemistry, hepatocellular oxidative stress in healthy and NASH liver samples. The proportion of hepatocellular 8-OHdG was higher in liver biopsies with NASH than in normal ones (NASH vs. control 64 vs. 37%, respectively, $p < 0.05$). They found no correlation between 8-OHdG expression and histological findings of steatosis, necroinflammation, ballooning, or fibrosis, which translates into the possibility of steatohepatitis, even in biopsies reported as normal; in this study, they also found a positive association between nuclear Nrf2 expression with hepatocellular 8-OHdG levels in NASH patients (r: 0.65, $p < 0.01$).[22]

ROLE OF OXIDATIVE STRESS IN THE PATHOPHYSIOLOGY OF NON-ALCOHOLIC STEATOHEPATITIS

All that is known about the pathophysiology of NASH has allowed the formulation of the *"multiple hits"* theory, in which dietary, genetic, and epigenetic factors, together with obesity, lead to elevated serum levels of free fatty acids and cholesterol, developing insulin resistance, adipocyte proliferation and changes in the intestinal microbiome. Insulin resistance in tissues

worsens adipocyte dysfunction, induces lipolysis, and promotes the release of adipokines and proinflammatory cytokines such as TNF-α and IL-6; which contributes to the maintenance of the insulin-resistant state. In the liver, increased free fatty acids and changes in the microbiome lead to increased triglyceride synthesis and accumulation, along with mitochondrial dysfunction due to increased oxidative stress and oxygen-free radical production, which promotes hepatic inflammation. Another important related factor is the gut-liver axis. Some studies have shown that the consumption of high-fat diets impairs the intestinal barrier. Increased permeability of the small intestine allows the translocation of molecules that contribute to inflammasome activation and increases endoplasmic reticulum stress.[21] Fatty liver shows increased susceptibility to lipid peroxidation with increased reactive oxygen species in response, causing mutations in DNA. In addition, some genetic factors play a role in pathogenesis. Variations in proteins such as adiponutrin (PNPLA3) and transmembrane superfamily 2 transmembrane 6 (TM6SF2) have been implicated in alterations in lipid metabolism and increased hepatic steatosis. The liver of patients with obesity is more susceptible to carcinogenesis as a result of impaired ATP production, coupled with a defective apoptosis mechanism and energy dysregulation and/or hormonal balance, hypoxia, and systemic inflammation.

Both endoplasmic reticulum stress and mitochondrial dysfunction generate apoptosis and fibrosis, processes that lead to the development of hepatic steatosis and NASH. An impaired autophagic function may be a causal factor in the development of hepatocarcinoma in patients with non-alcoholic steatohepatitis. Under normal conditions, it functions as a cell death mechanism and is also an adaptive mechanism to damage. Likewise, autophagy controls the breakdown of lipids contained in hepatocellular deposits; due to this, its deterioration can cause hepatic steatosis and the inability to inhibit the growth of tumor cells.[23]

The oxidative stress signaling pathway involves proteins such as *inositol-requiring enzyme 1α* (IRE1α), RNA, and endoplasmic reticulum kinases (PKR and PERK, respectively), as well as activated transcription factor 6α (ATF6α). When these molecules sense elevated levels of stress in the endoplasmic reticulum, they activate protein-mediated compensatory mechanisms. Stress can trigger the cell death cascade. In addition, chronic stress in the endoplasmic reticulum produces more reactive oxygen species that trigger hepatocyte inflammation through the nuclear factor κβ (NF-κβ) and Jun-N-terminal kinase (JNK) pathways.[21,24]

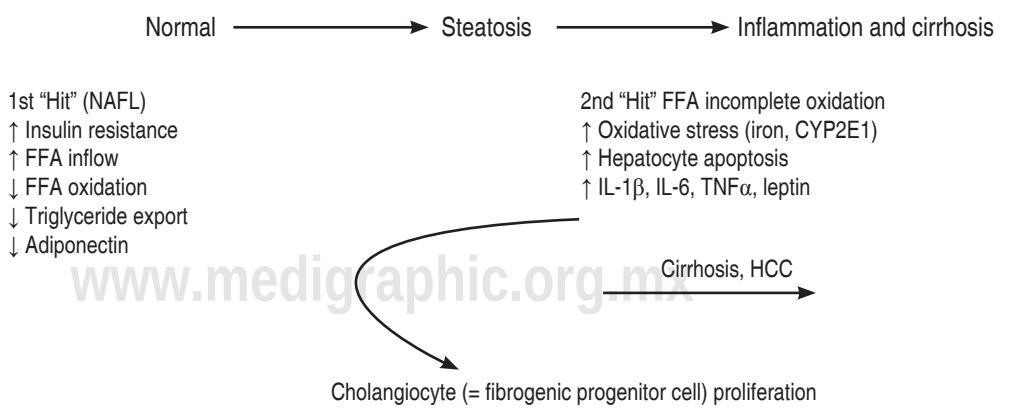


Figure 4: *Pathogenic mechanisms during progression to hepatic steatosis and non-alcoholic steatohepatitis. NAFL = non-alcoholic fatty liver. HCC = hepatocellular carcinoma. FFA = free fatty acids.*

DUAL ROLE OF THE KEAP1/ NRF2 COMPLEX IN LIVER CARCINOGENESIS

Nrf2 participates in several biological functions, such as metabolism and regulation of the response to xenobiotics, autophagy and apoptosis, pentose synthesis and NADPH generation, maintenance of the Redox balance, lipogenesis, cholesterol synthesis, gluconeogenesis, glycolysis, oxidation, and degradation of fatty acids. It also plays a fundamental role as a cytoprotective transcription factor by activating the cellular antioxidant response, thus being considered the main mechanism for cell survival and tumor suppressor. However, many studies point out that it may promote not only normal cell survival but tumor cells as well, resulting in the progression of neoplastic disease. Hyperactivation of Nrf2 also protects the tumor cell from oxidative stress, chemotherapy, and radiotherapy. Under physiological conditions, the Nrf2 pathway is stimulated by elevated oxidative stress and is inhibited as soon as the stimulus disappears; but under pathological conditions, the regulation of Nrf2 is altered, making the cell vulnerable to damage. Experimental studies in mice without Nrf2 show an increased incidence of neoplasms and metastasis with exposure to tobacco smoke, pentachlorophenol, and paracetamol as oncogenic stimuli.[25]

On the other hand, multiple genetic mutations associated with the disruption of the signaling pathway have been found to promote the progression of hepatocellular carcinoma. In a study of samples from 87 patients diagnosed with hepatocellular carcinoma, recurrent mutations were found in 8% of the genes transcribing for KEAP1. Other associated mutations are TP53 in 18%, CTNNB1 in 10%, C16orf62 in 8%, and MLL4 in 7 and 5% in the RAC2 gene.[25,26] Regarding Nrf2, a study identified the NFE2l2 mutation in 6.4% of a group of 125 patients with hepatocellular carcinoma.[27]

A cohort study of 107 patients with hepatocarcinoma followed for six years found that reduced KEAP1 expression in hepatocyte cytoplasm and nuclear overexpression of Nrf2 (Nrf2+/KEAP1-) is associated with increased recurrence and worse prognosis with only 40% disease-free survival at 80 months of treatment with liver resection.[28,29]

CONCLUSIONS

Oxidative stress plays a fundamental role in the pathophysiology of fibrosis, cirrhosis, and carcinogenesis in the liver. Nrf2 has a dual role; on the one hand, it functions as a major cytoprotectant regulating the synthesis of antioxidant enzymes; on the other hand, overexpression protects not only healthy cells but also tumor cells from oxygen-free radical attack, favoring tumor growth, recurrence, as well as chemo- and radio-resistance, being this duality the object of study, for the pathophysiology of other liver diseases and as a fundamental piece for the diagnosis, prognosis, and treatment at the molecular level of NASH and its progression.

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