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REVIEW ARTICLE

Gene Silencing using siRNA for Preventing Liver Ischaemia-Reperfusion Injury

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Abstract: ***Background*:** Ischaemia-reperfusion injury (IRI), a major complication occurring during organ transplantation, involves an initial ischemia insult, due to loss of blood supply, followed by an inflammation-mediated reperfusion injury. A variety of molecular targets and pathways involved in liver IRI have been identified. Gene silencing through RNA interference (RNAi) by means of small interference RNA (siRNA) targeting mediators of IRI is a promising therapeutic approach.

***Objective*:** This study aims at reviewing the use of siRNAs as therapeutic agents to prevent IRI during liver transplantation.

***Method*:** We review the crucial choice of siRNA targets and the advantages and problems of the use of siRNAs.

***Results*:** We propose possible targets for siRNA therapy during liver IRI. Moreover, we discuss how drug delivery systems, namely liposomes, may improve siRNA therapy by increasing siRNA stability *in vivo* and avoiding siRNA off-target effects.

***Conclusion*:** siRNA therapeutic potential to preclude liver IRI can be improved by a better knowledge of what molecules to target and by using more efficient delivery strategies.

Keywords: ???????????

1. INTRODUCTION

Liver transplantation is the standard of care for patients with end-stage liver disease and for those with hepatic tumours [1]. During the harvesting and preservation of the graft and during surgery cellular damage can occur, in a process that is known as ischaemia-reperfusion injury (IRI). Liver IRI is a clinically relevant condition affecting graft recovery and function and, ultimately, the success of liver transplantation [2].

The pathophysiology of IRI has been comprehensively studied but, despite its clinical importance, the mechanisms and cellular components involved in organ IRI are only partially understood due to their complexity [2-7]. This has hampered the establishment of adequate targets for effective therapeutics against IRI.

In this review, we first focus on the current knowledge of mechanisms triggering local immune activation and inflammatory cascades leading to cellular damage during liver IRI. Then we review the advantages and limitations of siRNAs as therapeutic agents. Finally, potential targets for the use of siRNAs in liver ischaemia-reperfusion injury and the use of drug delivery systems to overcome the limitations of siRNAs as therapeutic agents are also reviewed.

2. MECHANISMS OF LIVER ISCHAEMIA-REPERFUSION INJURY

Liver injury due to ischaemia–reperfusion (IR) can be divided into two major types [2]. The first is 'warm' ischaemia–reperfusion injury (IRI) which develops *in situ* during liver transplantation surgery. The second is 'cold' IRI which occurs during *ex vivo* liver preservation and is usually coupled with warm IRI during liver transplantation surgery. Hepatocytes are more sensitive to warm ischemia, whilst liver sinusoidal endothelial cells (LSEC) are more sensitive to cold ischemia which has as an outcome hepatic endothelium damage and microcirculation disruption [5, 6]. However, in both types of IRI immunological cascades involving the activation of Kupffer cells (KC) and neutrophils, the production of cytokines and chemokines, the formation of reactive oxygen species (ROS), the increased expression of adhesion molecules and infiltration by circulating lymphocytes and/or monocytes, occur [2, 6].

During ischaemia lack of oxygen supply in hepatocytes causes glycogen consumption, ATP depletion, higher rates of glycolysis, and alterations in H^+, Na^+ and Ca^{2+} homeostasis leading to cellular swelling [5]. Also, redox changes and ATP deficiency cause dysfunctions of key intracellular organelles such as mitochondria and trigger stress responses, e.g. the endoplasmic reticulum (ER) stress response [8] (Fig. **1**). The unfolded protein response (UPR) is activated upon ER stress and three ER transmembrane receptors, protein kinase R (PKR)-like endoplasmic reticulum kinase (PERK), activating transcription factor 6 (ATF6) and inositol requiring kinase 1 (IRE1), are involved in a signalling cascade that inhibits new protein synthesis and activates transcription of genes encoding proteins involved in protein folding and protein degradation in the ER [8]. Hypoxia also leads to the activation of the autophagy machinery to remove damaged organelles and ensure cell survival and limit cell death [6]. The final outcome is a low amount of hepatocyte death mainly by necrosis (although apoptosis can occur when ATP is less depleted) due to hypoxia and hyperosmotic swelling and also LSEC and EC swelling [5, 9]. Moreover, low nitric oxide

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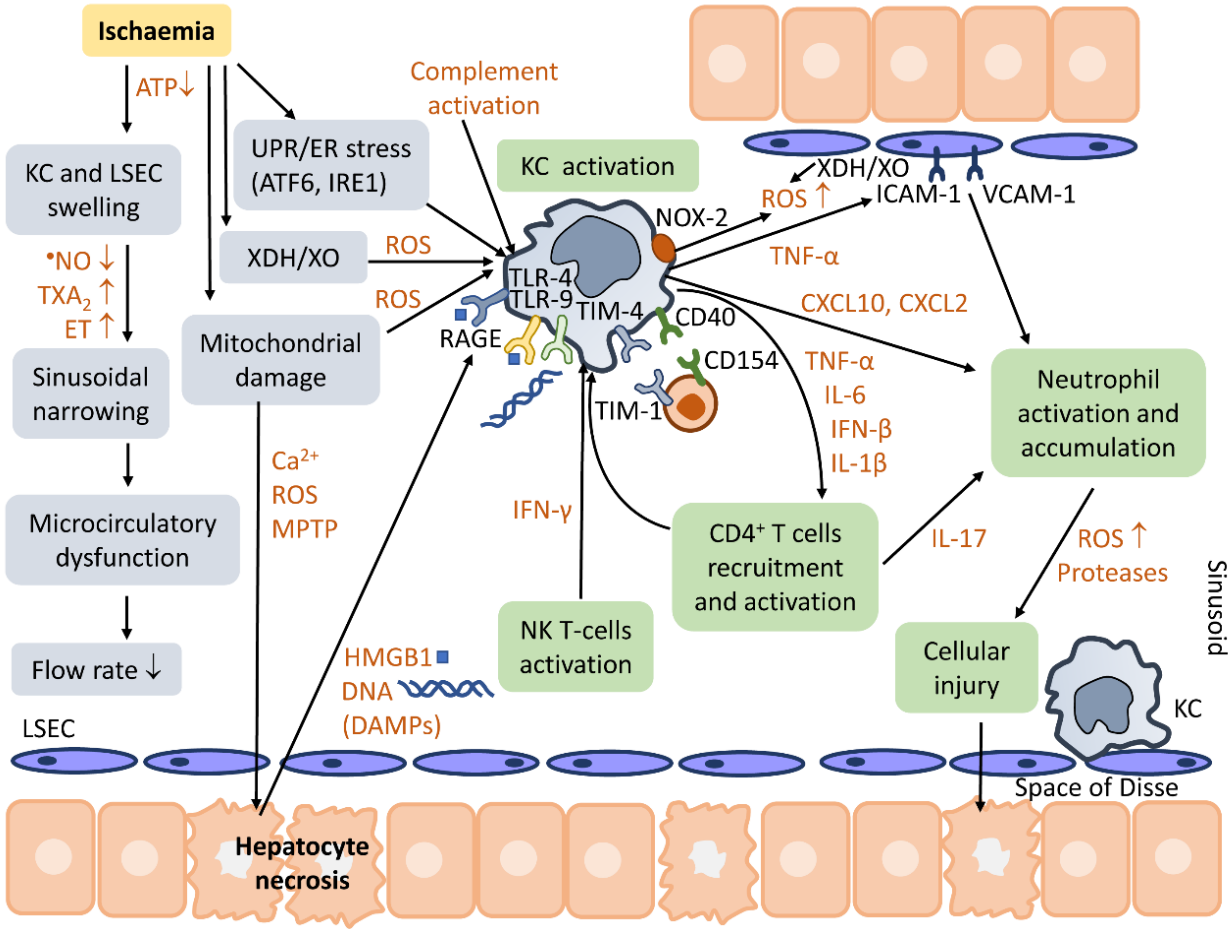

Fig. (1). Mechanisms underlying liver cold ischaemia and warm reperfusion injury. Ischaemia, due to lack of oxygen supply and ATP depletion, leads to microcirculatory dysfunction mitochondrial damage and hepatocyte death. Reperfusion leads to liver immune activation involving nonparenchymal liver cells (Kupffer cells, dendritic cells, natural killer cells) and is triggered by DAMPs released from necrotic cells, by activation of complement and by mitochondrial ROS production due to oxygenation. The recruitment of peripheral immune cells from the circulation (T cells and neutrophils) sustains the proinflammatory immune cascade activated by ischaemia-reperfusion which is responsible for the ultimate liver reperfusion injury. For a more detailed explanation see the main text. Hepatic stellate cells are not shown in the space of Disse and dendritic cells are also omitted, for the sake of clarity. ATF6, activating transcription factor 6; CD40, cluster of differentiation 40; CD154, CD40 ligand; CXCL2, C-X-C motif chemokine ligand 2; CXCL10, C-X-C motif chemokine 10; DAMPs, damage-associated molecular patterns; ER, endoplasmic reticulum; ET, endothelin; HMGB1, high mobility group box 1; ICAM-1, intercellular adhesion molecule 1; IL, interleukin; IRE1, inositol requiring enzyme-1; KC, Kupffer cells; LSEC, liver sinusoidal endothelial cells; INF, interferon; MPTP, mitochondrial permeability transition pore; NF-κB, nuclear factor kappa B; NK T-cells, Natural killer T-cells; NOX-2, NADPH oxidase 2; RAGE, receptor for advanced glycation end products; ROS, reactive oxygen species; TIM-1, T-cell immunoglobulin and mucin domain 1; TIM4, T-cell immunoglobulin- and mucin-domain-containing molecule 4; TLR-4, toll-like receptor 4; TLR-9, toll-like receptor 9; TNF-α, tumor necrosis factor α; TXA_2, Thromboxane A_2; UPR, unfolded protein response; VCAM-1, vascular cell adhesion molecule 1; XDH/XO, xanthine dehydrogenase/xanthine oxidase.

(•NO) levels and high levels of endothelin and thromboxane A2 contribute to a narrowing of the sinusoidal lumen and to microcirculation dysfunction [5]. The outcome is that on reperfusion the blood flow is significantly decreased, and some areas have a complete absence of blood flow, which is known as 'no-reflow' [10]. The hepatic endothelium damage occurring during cold preservation represents the initial factor leading to liver IRI [5].

Reperfusion injury, which follows ischaemic injury, is characterized by a sterile inflammatory immune response, involving KC, dendritic cells (DC), T cells, natural killer (NK) cells and neutrophils (for reviews see [7, 11]. Reperfusion injury can be divided into two phases [12], the first dominated by KC activation and the latter by neutrophil activation. In the early reperfusion phase, calcium overloading and increased reactive oxygen species (ROS) formation in the mitochondria cause mitochondrial dysfunction and the opening of the mitochondrial permeability transition pore (MPTP), leading to ATP depletion and necrotic hepatocyte death [13-15]. A potential source of ROS during liver reperfusion that has been extensively studied is xanthine oxidase (reviewed in [13]). In mammalian cells xanthine oxidoreductase (XOR) exists in two interconvertible forms, xanthine dehydrogenase (XDH), which is the predominant form in normal healthy tissue, and xanthine oxidase (XO). XO uses O_2 as the terminal electron acceptor generating superoxide radical ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2). In liver cells XOR expression is high [16] and the enzyme is also present at high levels on the outer surface of the plasma membrane of endothelial cells [17]. Studies in rats showed that XO levels in the circulation are significantly elevated following liver IR and it has been proposed that the enzyme is derived from plasma [18]. However, when trying to assess whether XDH is significantly converted to XO during liver IR, disparate results ranging from significant to no conversion of XDH have been obtained [13]. Moreover, during liver IR, XO seems not to be a major source of ROS production,

since hepatocellular injury response to I/R precedes the conversion of XDH to XO [19, 20]. However, production of $O_2^{\bullet-}$ can also occur via XDH which, under acidic conditions such as those occurring in ischaemia, has a NADH oxidase activity catalyzing the oxidation of NADH instead of xanthine [21]. Recently, it has also been shown that when the NAD pool is mainly reduced XDH is able to form large quantities of $O_2^{\bullet-}$[22].

Activation of KC is triggered by ROS, by complement (C3a, C5a, and MAC), a group of proteins that are involved in tissue injury and/or repair, and by damage-associated molecular patterns (DAMPs) [11, 23]. It leads to further formation of ROS, mediated by NADPH oxidase (NOX-2) formation of $O_2^{\bullet-}$, and to oxidative stress, which contributes to the early cell injury [11, 24] and to formation of cytokines which recruit the neutrophils that mediate the later portion of the injury [3]. The triggering of cytokine formation from KC also involves DAMPs, e.g. high-mobility group box-1 (HMGB1) protein, heat-shock proteins and DNA fragments, which are released from necrotic hepatocytes upon reperfusion and can stimulate pattern recognition receptors (PRRs) [25]. The two main classes of PPRs involved in the IRI inflammatory response are toll-like receptors (TLRs) and the receptor for advanced glycation end products (RAGE) [2, 10]. HMGB1, a nuclear protein, has been identified as an endogenous TLR-4 ligand with a key role in innate immune activation during IRI [26], whilst DNA fragments are ligands for TLR-9 [27]. HMGB1 extracellular release is triggered by TLR-4-dependent ROS formation, probably H_2O_2. This is followed by the nuclear translocation of the transcription factor interferon regulatory factor 1 (IRF-1), leading to the up-regulation of histone acetyltransferase (HAT) activity, acetylation of HMGB1 and its extracellular release [11]. HMGB1 released in the circulation is scavenged by either TLR-4, expressed in KC, DC, and to a lesser degree in hepatocytes and LSEC, or RAGE, expressed in DC, KC and neutrophils and monocytes [11, 28-30], which leads to the activation of downstream signalling cascades. Ligation of TLR-4 by HMGB1 is MYD88-independent and involves recruitment of adapters TRAM and TRIF to induce the translocation of IRF-3 to the nucleus and the formation of IFN-β [31]. Alternatively, ligation of HMGB1 to RAGE activates mitogen activated protein kinases (MAPKs) and leads to the EGR-1-dependent formation of pro-inflammatory cytokines such as TNF-α and chemokines CXCL10 and CXCL2 [28].

During the early phase of reperfusion injury TNF-α, a central mediator in hepatic inflammatory response to IR and oxidative stress, activates NF-κB in KC, hepatocytes and endothelial cells [2, 32]. NF-κB activation in KC upregulates TNF-α and IL-6 which leads to the activation of $CD4^+$ T lymphocytes and, to a lesser extent, of NK T-cells. $CD4^+$ T lymphocytes operate as inflammatory signal amplifiers activating KC, and also as facilitators of neutrophil recruitment which leads to increased levels of chemotactic messengers [2, 11, 33]. In fact, CD154 and T-cell immunoglobulin and mucin domain 1 (TIM-1) proteins present at the surface of $CD4^+$ T cells associate respectively with CD40 and TIM-4 present at the surface of KC and LSEC. CD40 recruits TNF receptor associate factor 6 (TRAF6) and Src kinase to activate KC. TRAF6 activates NF-κB, ERK and p38 MAPK which leads to production of cytokines (e.g. IL-1 and TNF-α), chemokines (e.g. CXCL8, CXCL2) and $^{\bullet}NO$ by KC and LSEC. Release of the proinflammatory cytokine IL-17 by $CD4^+$ T cells also leads to expression of other proinflammatory cytokines including IL-6, TNF-α, and IL-1β and chemokines, particularly the CXC chemokines, partly through activation of NF-κB [34]. Other inflammatory cytokines such as IL-12 and IL-23, possibly produced by KC and stellate cells, are also involved in the early inflammatory response by stimulating $CD4^+$ T cells and γδT-cells to produce IL-17 and by activating NF-κB. Activated NK T-cells release IFN-γ which promotes formation of chemokines by KC, and promotes, through the transcription factor STAT1, the increased expression of adhesion molecules at the surface of LSEC [35].

The late phase of IRI is characterized by recruitment of neutrophils and damage to hepatocytes promoted by neutrophils through activation of NADPH oxidase (NOX-2), leading to $O_2^{\bullet-}$ release and formation of other ROS (H_2O_2, HOCl, $ONOO^-$), and through release of proteases during degranulation. Neutrophil recruitment requires chemotactic agents and vascular adhesion molecules. NF-κB activation in LSEC leads to TNF-α up-regulation and to TNF-α-dependent up-regulation of CXC chemokines and of adhesion molecules such as E-selectin, ICAM-1 and VCAM-1 [36, 37]. Proinflammatory mediators can also be released by inflammasomes which sense the presence of necrotic cells [38]. NLRP3, a member of the NOD-like receptor family of PRRs, can be activated and give rise to the NLRP3 inflammasome and is involved in the mechanism of neutrophil recruitment to sites of focal hepatic necrosis. NLRP3 silencing decreases the levels of IL-1β, IL-18, IL-6, TNF-α and HMGB1 and attenuates IRI [38]. During the reperfusion phase hepatocyte death can be massive and occurs mainly by necrosis [39, 40].

3. siRNAs AS THERAPEUTIC TOOLS

At the end of the 20th century, it was found that exogenously introduced double stranded RNA (dsRNA) molecules and plasmids expressing short hairpin RNA (shRNA) were able to specifically do base-pairing with target mRNA molecules causing their degradation (RNA interference, RNAi) [41, 42]. These studies exposed the existence in eukaryotic cells of specific silencing pathways based on small non-coding RNAs (sncRNAs). RNAi is mechanistically related to a number of other conserved RNA silencing pathways that evolved as important regulators of gene expression and genome stability by protecting it against virus, mobile repetitive DNA sequences, retro-elements, and transposons [43]. Three major classes of RNA silencing pathways operating in eukaryotic cells can be defined based on the mechanism of action, subcellular location and the biogenesis pathways of the small RNA molecules involved, *i.e.* short interfering RNAs (siRNAs), microRNAs (miRNAs) and PIWI-interacting RNAs (piRNAs). siRNAs are small RNA duplex molecules produced by the action of Dicer, a ribonuclease III (RNaseIII) enzyme that creates RNA duplexes with 2-nt overhangs at their 3' ends and phosphate groups at their 5' ends [43]. The discovery of RNAi led to the development of the RNAi technique that uses synthetic siRNAs, 21–23 nt in length to transfect mammalian cells in culture to specifically suppress the expression of endogenous genes. In the last years, this method has been explored as a powerful tool to determine biological functions of genes and soon emerged as a potential therapeutic approach to silence disease-related genes. In fact, a significant number of siRNA-based therapies are already in development. A search for siRNA at the NIH clinical trials database (https://www. clinicaltrials.gov, May 2018) gets 56 clinical trials either performed or currently on going. In these clinical trials several diseases have been targeted, e.g. cancer, viral infections, inflammatory disorders, cardiovascular disorders, neurological disorders, ocular disorders and metabolic disorders (for review [44]).

siRNAs attractiveness as a new class of therapeutics is due to improved rational design strategies and selection algorithms developed in the last years, which allow to careful select their sequences to potentially downregulate every single gene with diminished off-target effects [45, 46]. Also, siRNA can be specifically targeted to different transcripts of a gene, splice variants and mutations in transcripts and used at lower concentrations when compared to other antisense oligomers or ribozymes. However, the effectiveness of the knockdown caused by siRNAs is dependent on the target sequence positions selected from the target gene [47], and a number of siRNAs have been shown to be non-functional or to have low efficacy in mammalian cells [47, 48].

Despite siRNAs potential for therapy there are still some major challenges that should be overcome (Fig. **2**). These challenges probably explain why, despite the number of patents and clinical trials, as far as we know, no siRNA-based therapeutic products have reached the market [44, 49]. One of the problems of using siRNAs as therapeutic agents is the fact they do not discriminate cell types and tissues causing a global gene silencing [50]. Another difficulty is due to the fact that siRNAs are either rapidly eliminated by the kidneys due to their small molecular mass or captured by the phagocytic cells of the mononuclear phagocytic system (MPS) [51]. Furthermore, they are rapidly degraded by endogenous RNases in the plasma before cellular internalization which is disadvantageous for prolonged expression of RNAi-based therapeutics [52, 53]. siRNAs also have poor cellular uptake since, being big macromolecules and relatively heavily charged, they are not able to cross cell membranes by diffusion [54]. Consequently, the endosomal pathway is a privileged entry pathway for siRNAs. However, siRNAs may be degraded or remain trapped inside of endosomes which compromises their role inside the cell [55].

Activation of the immune response by siRNAs in a sequence- and concentration-dependent manner is also an obstacle for their use as therapeutics [58]. Several studies showed that certain siRNA sequences trigger immune activation via the TLR-3/7/8 and PKR cascades leading to the activation, for example, of genes coding for interferons, pro-inflammatory cytokines (*e.g.* TNF-α), chemokines and chemokine receptors [59-63]. Nevertheless, the fact that siRNAs can trigger sequence- and target-independent angiogenesis through TLR-3 [64] shows that off-target effects of siRNAs when used as therapeutic agents could be more complex and are not yet completely understood. Another concern comes from the fact that siRNAS and miRNAs share their biogenesis and mechanism of action. Thus, increased levels of a siRNA may affect endogenous miRNAs, their regulatory functions, and become toxic. This is supported by the observation that miRNAs in hepatocytes are down regulated upon delivery of high levels of shRNA expression specific for six targets using an adeno-associated virus (AAV)-based gene delivery system, which caused mice morbidity [65].

4. POTENTIAL TARGETS FOR THE USE OF siRNAs IN LIVER ISCHAEMIA-REPERFUSION INJURY

In liver transplantation direct siRNA therapeutics aimed at improving the quality of the graft before surgery, and without having

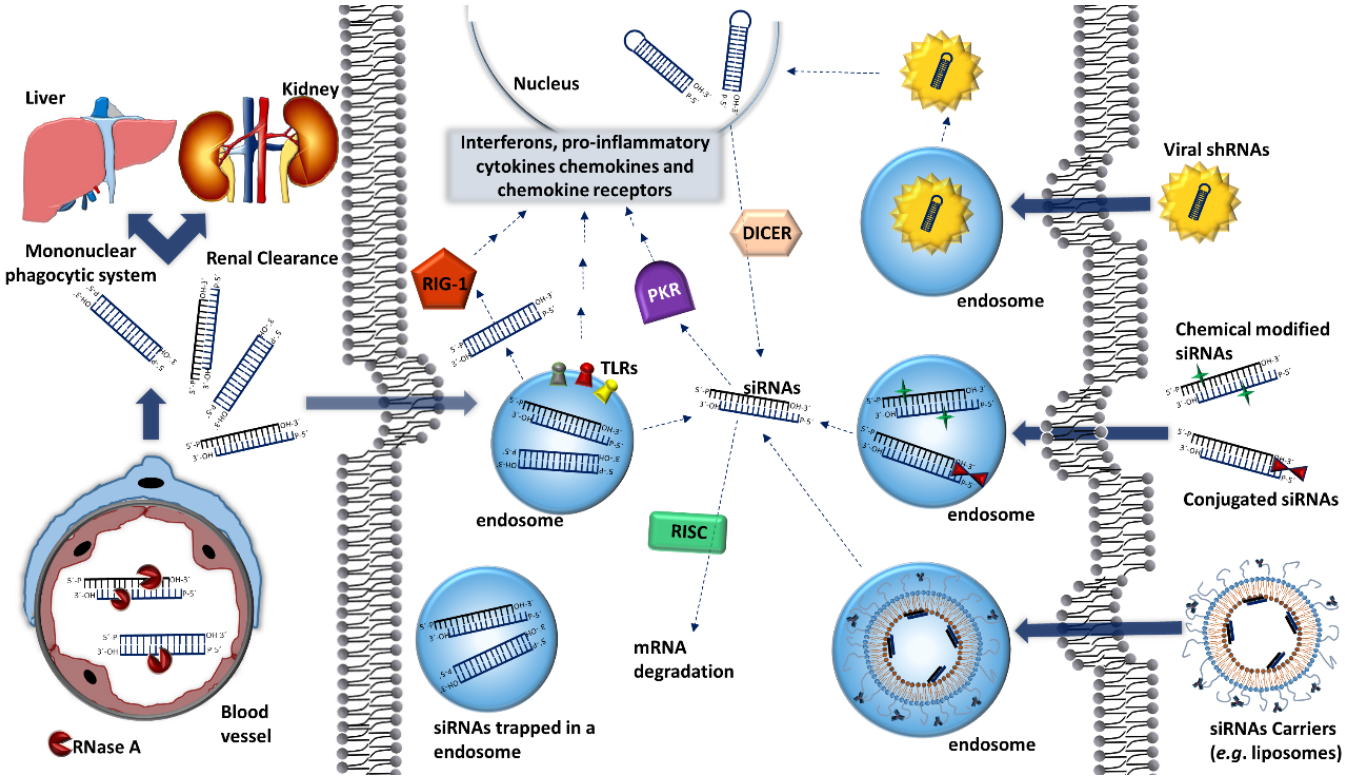

Fig. (2). Problems and strategies for delivering siRNAs. Main delivery drawbacks of siRNAs: siRNAs have non-favorable pharmacokinetic properties since they are rapidly eliminated by the kidneys due to their small molecular mass and are captured by the mononuclear phagocytic system. In the blood system they are rapidly degraded by endogenous RNases before cellular uptake and, due to being large macromolecules heavily charged, they display weak transport across cell membranes. The endosomal pathway is a privileged via of entry for siRNAs, but they may be degraded or entrapped within endosomes. siRNAs released into the cytoplasm via endosomes are incorporated into RISCs (RNA-induced silencing complex) to guide the recognition and degradation of specific target mRNAs. In endosomes certain siRNA sequences activate an immune response that may occur through different pathways: (i) recognition of siRNAs by TLRs; (ii) activation of retinoic acid-inducible gene I (RIG-I) by blunt-end siRNAs; (iii) activation of dsRNA-dependent protein kinase R (PKR). Strategies for effective siRNAs delivery - Introduction of siRNA chemical modifications or siRNA conjugation strategies with hydrophobic ligands (cholesterol, α-tocopherol), peptide fusion proteins, membrane permeable peptides (penetratin or transportan) and aptamers decrease siRNA susceptibility to nuclease activity, enhance cell uptake, lower the incidence of off-target effects and increase pharmacodynamics without affecting the silencing efficiency of target genes. siRNA delivery carriers, e.g. cationic liposomes and polymers, such as PEG, form complexes with negatively charged siRNA and allow efficient cellular uptake by endocytosis. To enhance and/or extend gene silencing viral vectors can be used (adenovirus, retrovirus, lentivirus, or adeno associated virus) to deliver shRNA. Short-hairpin RNA is translocated into the cell nucleus, transported to the cytoplasm and processed by the Dicer RNase III into functional siRNAs. Adapted from [55-57].

to worry about off-target delivery of siRNA, can be performed by adding siRNAs to the perfusion medium used to preserve *ex vivo* liver during organ storage and transport to the transplant recipient. However, siRNA therapeutics can be used in other phases of liver transplant by developing nanosystems (nanoparticles/liposomes, etc.) for proper targeting and delivery. No studies have been done in liver transplantation using siRNAs in the perfusion media so far. However, recently, a similar strategy was used on a kidney transplant model. By perfusing kidneys with a siRNA cocktail solution targeting complement 3, RelB (one of the proteins of the NF-κB complex), and first apoptosis signal receptor (Fas) cold IRI injury was prevented [66].

As said previously, liver damage occurs both during ischaemia and reperfusion. One of the problems of using siRNA for IRI therapeutics is the choice of target, since IRI is a complex process involving several types of cells and signalling pathways which are not completely unravelled. In that respect, new mechanistic insights into the molecular events involved in IRI, leading to future therapeutic use, can be obtained by using siRNAs. For example, recently, the use of ATF6 siRNA in a murine warm ischaemia model allowed to show that ischaemia primes murine liver innate immune cells by ATF6-mediated ER stress response [67].

Initial studies using siRNA specific to selective gene sequences which play a key role in hepatic IRI, were targeted mainly at apoptosis. First apoptosis signal receptor (Fas) knockdown by siRNA led to lower serum alanine aminotransferase (ALT) levels, a biomarker of liver damage, after IR [68] whilst *in vivo* knockdown by siRNA of acidic sphingomyelinase [69] decreased ceramide generation during IR, and attenuated serum ALT levels, hepatocellular necrosis, cytochrome *c* release, and caspase-3 activation. However, more recent studies have shown that both apoptosis and necroptosis have a minor role in hepatocyte death during IRI [39, 40, 70].

A good therapeutic strategy to prevent liver IRI is to use as preferential targets known upstream mediators of KC activation and the proinflammatory process involved in reperfusion injury. Therefore, good candidates for the use of siRNA to prevent KC activation are key mediators involved in TLR- or RAGE- triggered inflammatory signal pathways. Among them, IRF1 [71, 72], HMGB1 [73] or other DAMPs [74], complement receptors [75], TLR-4 [76], RAGE [30] or TLR-9 [27]. Recently, HMGB1-siRNA was used therapeutically to reduce 60-70% nuclear HMGB1 expression in mice liver and then mice were subjected to liver IR [73]. HMGB1-siRNA pretreatment markedly inhibited HMGB1 release after hepatic reperfusion and the increases in hepatic expression of TLR-4, TLR-2, RAGE, TNF-α, IL-1β, IL-6, monocyte chemoattractant protein 1 (MCP-1), inducible nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX-2) seen in control mice after hepatic reperfusion. Also, there was a significant preservation of liver function and a marked decrease in liver damage when compared to control mice. TLR-4 siRNA treatment of liver IRI has been tested *in vivo* using a hepatocyte-specific delivery system consisting of galactose-conjugated liposome nanoparticles (Gal-LipoNP) [76]. TLR-4 siRNA treatment significantly decreased serum ALT and aspartate transaminase (AST) and histopathology displayed an overall reduction of the injury area in the Gal-LipoNP TLR-4 siRNA treated mice. Additionally, there was a suppression of the inflammatory cytokines IL-1 and TNF-α and neutrophil accumulation and lipid peroxidation-mediated tissue injury were attenuated after Gal-LipoNP TLR-4 siRNA treatment. In a recent study it was shown that the use of RAGE siRNA alleviated liver injuries and inhibited inflammatory immune activation against IR in diabetic, but not normal, mice [30].

Two other mediators with a key role in IRI worth targeting with siRNA silencing are TNF-α and NF-κB. Silencing of TNF-α expression with shRNA during liver IR led to a decrease in IRI [77]. The use of siRNA to silence TNF-α expression in a renal model also decreased IRI [78]. No studies have been made in liver IRI to prevent activation of NF-κB or silence the transcription factor**.** However, in renal IRI models, targeted silencing of IκB kinase β (IKKβ) [79] or RelB [59] to prevent NF-κB activation using siRNA substantially attenuates kidney injury and inflammation following ischemia-reperfusion.

Since the later part of IRI involves recruitment of neutrophils and further amplification of inflammatory damage to liver cells, strategies aimed at decreasing the expression of adhesion molecules (e.g. ICAM-1, VCAM-1, P-selectin) by using siRNA will probably also decrease IRI as shown in [80]. Another important target for the use of siRNA should be NOX-2 which is involved both in the early and later phases of IRI through its activation in KC and neutrophils. Specific inhibitors of NOX-2 are lacking and it has been shown in mice models of myocardial infarction that delivery of Nox-2 siRNA with polyketal nanoparticles prevents up-regulation of Nox-2 and significantly recovered cardiac function [81].

5. OVERCOMING OF THERAPEUTIC LIMITATIONS siRNAs FOR ISCHAEMIA-REPERFUSION INJURY TREATMENT

Vast efforts have also been made to improve siRNAs delivery for therapeutic use in order to simultaneous protect siRNAs during transport and prevent non-specific delivery and promote delivery to target tissues/cells [55, 82, 83]. Even if the degradation by serum RNases could be surpassed, naked siRNAs would have a very low transfection efficiency due to their low cellular internalization because of their physico-chemical characteristics [84]. Several strategies have been developed to overcome siRNAs limitations, e.g. by structurally modifying siRNAs [55] introducing chemical modifications at the ribose sugar backbone (*e.g.* 2′-fluoro, Locked Nucleic Acids (LNAs contain a methylene bridge which connects the 2′-O with the 4′-C of the ribose), 2′-O-methyl RNA (2′OMe), 2′-fluoro-β-D-arabinonucleotide (FANA) and 2′-O-(2-methoxyethyl) RNA 2′(MOE)), and phosphodiester backbone (*e.g.* phosphorothioate, boranophosphate, and methylphosphonate) of siRNA molecules [85, 86]. Many of these modifications decrease siRNA susceptibility to nuclease activity, lower the incidence of off-target effects and increase pharmacodynamics without affecting the silencing efficiency of target genes. Similarly, strategies involving conjugation of siRNAs, namely with hydrophobic ligands (cholesterol, α-tocopherol) and polymers such as polyethylene glycol (PEG) [87] improved their pharmacological properties by increasing circulation half-life and enhancing cellular uptake [55, 83, 88]. The use of nonpolymeric or polymeric drug delivery systems (DDS) such as liposomes, self-assembly phospholipid carrier, polyplexes complexes solid lipid nanoparticles, polymeric nanoparticles, nanoemulsions, etc. has also been a successful approach to deliver siRNAs to target organs/cells [89-92].

One of the first reports of the use of a drug delivery system to treat ischaemic tissue was published in the early 1980s. Palmer *et al.* [93] showed that liposomes accumulated in ischaemic tissues (myocardium) and that there was an inverse linear correlation between liposomal distribution and regional myocardial blood flow. Although, the mechanism of liposome accumulation was not known, it was proposed they were behaving as microprobes sensitive to the biochemical environment and responding to changes in this environment by specific and non-specific structural alterations. After the work of Matsumura and Maeda [94], the mechanism of accumulation of nanoparticles was elucidated and now it is well established that it involves the enhanced and retention effect (EPR), due to enhanced vascular permeability occurring in tissues in situations such as in inflammation. The *in vivo* fate of nanosystems drugs by EPR for the treatment of inflammations such as rheumatoid arthritis is now well established [95-99]. It has been proved that particles with a size lower than 0.15 μm and with high circulation time (>15-20h) accumulate preferentially at inflamed sites. This process is known as passive targeting. As an example, in a

mouse cerebral artery occlusion model and in the case of liposomal antioxidant (superoxide dismutase) delivery, it was shown the ability of passively targeted nanoparticles to be effective in the reduction of infarct volume and improvement in behaviour after cerebral ischemic injury [100].

In liver IRI, there seems to be a preferential accumulation of long circulating (PEGylated) and small size (<0.150 nm) liposomes at the sites of inflammatory-type lesions. In fact, magnetoliposomes with a negative contrast agent (SPION) and the same characteristics improved the visualization of the injuries caused by IR [101]. The liposomal uptake by the liver and their intra-hepatic distribution [102] has been attributed to the characteristics of the liposomal formulation in terms of stealth and size properties [97, 103]. The same type of PEGylated and small size liposomes with an anti-inflammatory associated carrier has been used to treat hepatic ischaemia-reperfusion lesion and showed an effective outcome in terms of therapeutic activity [98, 104]. Another example is the use H_2O_2-triggered bubble-generating antioxidant polymeric PVO nanoparticles as I/R passive targeted nanotheranostic agents. PVO nanoparticles significantly enhanced the ultrasound contrast in the site of H_2O_2- accompanying hepatic I/R injury and remarkably inhibited the liver damages and apoptotic cell death [105]. These studies show that nanosystems can be used as a delivery system to passively target siRNAs to the IR liver overcoming the drawbacks of naked siRNA by increasing its short plasma half-life, protecting from its enzymatic degradation and modifying its biodistribution.

In addition to passive targeting, surface modification of the drug delivery systems can be done in order for them to be recognized by specific cell receptors allowing a direct interaction with the target cells, a process known as active targeting. Moreover, active targeting allows intracellular delivery through receptor-mediated internalization. Specific cell targeting molecules (antibodies, aptamers, and ligands for cell surface receptors) which enable the recognition of specific types of cells have been extensively investigated and tested [106].

One of the few and first works using siRNA silencing to treat liver IR injuries was published in 2011 and used a liver-specific liposome-based siRNA delivery systems using PEGylated liposomes with active targeting to galactose receptors encapsulating TLR-4-siRNA. This system efficiently knocked down TLR4 gene synthesis in liver and attenuated liver IRI, protected liver function, decreased neutrophil infiltration and suppressed inflammatory cytokines [76]. Other examples of the use of nanosystems with special attention paid to the effect of targeted delivery, lead to the conclusion that they can be clinically useful to treat liver IR with siRNA [95, 100].

As siRNAs are needed in the cytosol to achieve their therapeutic activity, an efficient release from the endosome is needed. A carrier system to efficiently perform a cytosolic delivery of siRNA should follow several general principles [92]. First, to stabilize siRNA, lipids/polymers with a positive charge are normally used since the work of Felgner *et al*. [107]. Nevertheless, a neutral net charge of the nanosystem will be required to avoid the interaction with the MPS and nonspecific cell-binding and to prolong its half-life [108, 109]. This can be achieved by coating the particle with PEG [97, 110]. Another important characteristic of the nanosystems must be the possibility of active targeting to specific receptors of cells involved in liver IR leading to uptake via the scavenging receptor [111] as for example with E-selectin ligands [112]. Moreover, escape from the endosome is mandatory for the cytosolic delivery of siRNA and this can be achieved using substances that will disrupt the endosome before siRNAs are degraded. Several approaches can be made but the more commonly used is based on the incorporation of ionizable cationic lipids with the capacity of destabilizing the bilayer as a function of pH to release siRNA from the endosome [90, 92, 111, 113].

CONCLUSION

Despite all its promising therapeutic outcomes, the use of siRNA as a therapeutic drug has been hindered by several limitations. As shown in this review, siRNA has been used for IRI but its potential can be improved by a better knowledge of what molecules to target and also with better delivery strategies. Here, with the aim of using siRNAs as therapeutic drugs to prevent liver IRI during transplantation we proposed several targets for the use of siRNAs, analysed the advantages and problems of the use of siRNAs, and showed what types of drug delivery systems are able to improve siRNA therapeutics.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The author(s) declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.
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