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REVIEW

Novel strategies for the treatment of diabetic macular edema

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Abstract

Macular edema such as diabetic macular edema (DME) and diabetic retinopathy are devastating back-of-the-eye retinal diseases leading to loss of vision. This area is receiving considerable medical attention. Posterior ocular diseases are challenging to treat due to complex ocular physiology and barrier properties. Major ocular barriers are static (corneal epithelium, corneal stroma, and blood-aqueous barrier) and dynamic barriers (blood-retinal barrier, conjunctival blood flow, lymph flow, and tear drainage). Moreover, metabolic barriers impede posterior ocular drug delivery and treatment. To overcome such barriers and treat back-of-the-eye diseases, several strategies have been recently developed which include vitreal drainage, laser photocoagulation and treatment with biologics and/or small molecule drugs. In this article, we have provided an overview of several emerging novel strategies including nanotechnology based drug delivery approach for posterior ocular drug delivery and treatment with an emphasis on DME.

Key words: Diabetic macular edema; Photocoagulation; Retinopathy; Biologics; Vitrectomy; Corticosteroids; Nanoformulations; Laser

Core tip: Macular edema such as diabetic macular edema (DME) and diabetic retinopathy are devastating back-of-the-eye retinal diseases leading to loss of vision. The standard treatments of DME include laser photocoagulation, vitrectomy, intravitreal injections of anti-vascular endothelial growth factor biologics and steroids. In this article we have provided an overview of several emerging novel strategies including nanotechnology based drug delivery approacher for posterior ocular drug delivery and treatment with emphasis on DME.

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INTRODUCTION

Diabetic macular edema (DME) is a chronic back-of-the-eye disease that may lead to vision loss. DME causes retina thickening due to accumulation of fluid in the center of macula (Figure 1)[1]. Chronic diseases such as diabetes, non-proliferative and proliferative diabetic retinopathy (DR) are significant factors for the onset of DME[2,3]. The exact mechanism by which diabetes leads to retinopathy is not well-delineated. However, several theories have been postulated in the literature. DR may develop due to excessive growth of leaky vascularization in the retina. According to National Eye Institute, DR progresses in four stages[4]. In brief, stage 1 *aka* mild non-proliferative retinopathy, is the initial stage where tiny abnormal blood vessels or micro aneurysms are developed. Such blood vessels appear as balloon-like swelling in the retina. With disease progression, stage 2 *aka* moderate non-proliferative retinopathy ensures blockage of blood vessels that supplies nutrition to retina. Severe non-proliferative retinopathy *aka* stage 3 is diagnosed with blockage of blood vessels thereby depriving blood flow to the retina. Under such conditions retina lacks oxygen and nutrients supply. Moreover, several cellular signals (particularly HIF-α) are triggered that cause development of new vasculature to compensate oxygen and nutrient supply. Proliferative retinopathy is termed as the final stage or the advanced stage of DR. The new abnormal blood vessels developed are fragile, and leaky. Such development is termed as neovascularization. Several factors can add to severity of DME depending on the degree of DR, length of time the subject is diabetic, type of diabetes, hypertension, fluid retention, hypoalbuminemia and hyperlipidemia. Advent of microscopic techniques such as fundus contact lens bio-microscopy or funduscopic examination are proven to aid DME diagnosis. It can be diagnosed with ocular clinical conditions such as retinal thickening within 500 μm and/or hard exudates within 500 μm or in one disk diameter from the center of macula[5].

Pathogenesis of DME is not clearly delineated in the literature. However, DME is a complex multifactorial ocular disease[6]. Blood retinal barrier (BRB) is an essential structure that regulates normal visual function. Such a physiologic barrier also regulates fluid and solute movement in and out of retina[7]. BRB is comprised of inner and outer BRB[8,9]. The inner BRB is composed of tight junctions between retinal capillary endothelial cells while the outer BRB tight junctions exist between retinal pigment epithelial cells[7]. The breakdown of inner BRB results in vasogenic edema, neural tissue impairment and ultimately vision loss, if not treated[10]. Disruption of BRB is one of the common factors for DME development[11,12].

PHYSIOLOGY OF DME

Many macro and microvascular factors along with various pathways are involved in retinal thickening, disruption of BRB and loss of pericytes[13].

Macro-vascular factors

Macro-vascular factors include Starling's law for edema, oxygen tension and shear stress.

Starling's law and macular edema: According to the Starling's law, hydrostatic blood and osmotic pressures of tissue fluid are responsible for vasogenic edema. It appears to maintain the gradients between two forces involving fluid movement between inner and outer retinal layers which is crucial to prevent DME[10]. This law is based on water accumulation caused by decreasing osmotic pressure gradient between vessel and tissue. Current strategies for DME such as vitrectomy, laser, anti-vascular endothelial growth factor (anti-VEGF) or steroids can lower osmotic pressure gradient and vascular permeability to prevent water accumulation.

Oxygen tension: In diabetic patients, the level of oxygen is reduced in the macular region. Consequently hypoxia induces VEGF expression[14,15] resulting in enhanced vascular permeability. Elevation of oxygen tension causes compensatory vasoconstriction of the retinal vessels which can reduce hydrostatic pressure, resulting in macula edema[13,16,17]. Stefánsson[18] has explained how and why vitrectomy and photocoagulation can have effects on DME and other neovascularization retinopathies due to improved ocular oxygen tension.

Shear stress: The damage of endothelial cells and decoupling caused by shear stress over time can lead to alterative fluid flow in edema. Increase in shear stress also elevates nitric oxide (NO) production, which may result in vasodilatation and elevated hydrostatic pressure[19].

Microvascular factors

Endothelial dysfunction and vascular damage due to hyperglycemia: Endothelial cells play a vital role in maintaining the structure, vascular tone and prevention of platelet and leucocyte adhesion onto vessel wall. These cells are responsible for production of vasoconstriction, a dilatation and various inflammatory mediators such as intracellular adhesion molecule, leucocyte adhesion molecule, and vascular cell adhesion molecule[20-22]. While endothelial progenitor cells play a role in repair of damaged vessels, number of these cells are highly reduced under hyperglycemic conditions[23,24].

BRB: Since endothelial cells play an important role in maintaining the integrity of BRB, damage in endothelial cells leads to disruption and leakiness of vascular beds.

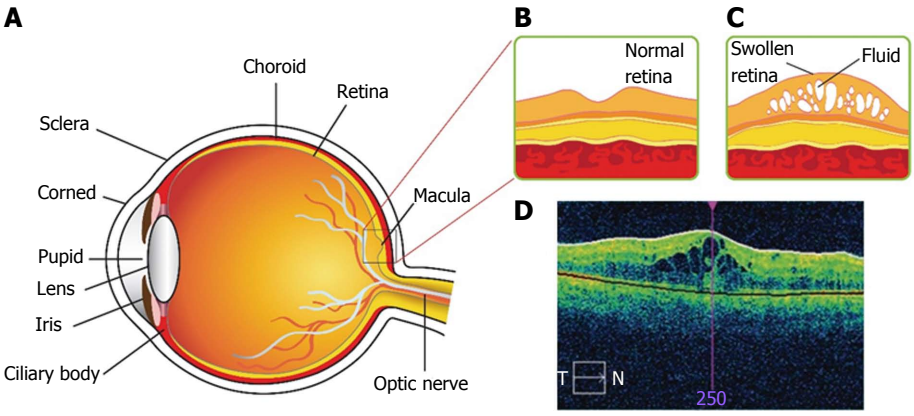

Figure 1 Diabetic macular edema at disease state. A: Structure of human eye; B: Expanded representation of macula region for normal eye; C: Expanded representation of macula region for diabetic macular edema (DME); D: Optical coherence tomography image for DME.

This increased permeability leads to accumulation of extracellular fluid. It increases the oncotic pressure due to influx of protein from blood vessels to inner retina[25,26].

Growth factors: Growth factors regulate angiogenesis by stimulating endothelial cell proliferation, migration, and survival. These factors have profound influence in many ocular diseases such as DME, DR and neo-vascular age-related macular degeneration (AMD)[27-29]. Growth factors including VEGF, placental growth factor, and hepatocyte growth factor are responsible for increased vascular permeability. VEGF appears to be an important factor for endothelial cell migration, proliferation and survival.

Inflammation: Inflammation plays a crucial role in DME pathogenesis. Leucocytes naturally adhere to vascular endothelium (leukostasis) and have the ability to create toxic superoxide radicals and enzymes[30]. Leukostasis induce vascular permeability and impair endothelial cells by producing enzymes, cytokine and free radicals[31,32]. Also inflammation motivates the occludin phosphorylation which regulates tight junction and barrier function resulting in the breakdown of BRB[33-35].

Oxidative stress: Diabetes can cause oxidative stress leading to elevated levels of NO, superoxide, peroxynitrite development and VEGF expression, all of which may alter vascular permeability and BRB breakdown[36-38].

Others factors include matrix metallo proteinases, protein kinase C, carbonic anhydrase, and angiotensin-Ⅱ that have direct or indirect role in enhancing vascular permeability that results in DME[9,39-42]. Moreover, several pivotal pathways have been implicated in DME such as angiogenesis, inflammatory and oxidative stress pathways[9,11,13,43]. Chronic hypertension and hyperglycemia cause blood vessels to become more porous allowing fluid, lipid sand erythrocytes escape. Such leakage and accumulation only cause vascular basement membrane thickening, free radical formation, non-enzymatic glycosylation and pericyte death[44]. Moreover, increased vascular permeability and capillary dropout may cause inadequate blood supply to retina.

EXISTING AND EMERGING DRUGS AND TREATMENT MODALITIES

The current treatment strategies for DME has been summarized in Figure 2 and discussed in detail as followings.

Laser photocoagulation

Despite the fact that anti-VEGF [bevacizumab, ranibizumab (RBZ) and pegaptanib] and VEGF trap (aflibercept) have emerged as treatment options for back-of-the-eye diseases, laser (focal or/and grid) photocoagulation surfaced as another treatment option for DME[45]. A recent study conducted on non-center involved (CI) DME subjects involves focal laser photocoagulation. In this study 29 eyes with non-CI received focal laser coagulation and 20 eyes with no treatment served as control. Photocoagulation treated eyes demonstrated a 5 letter gain in visual acuity in 21% subjects relative to 5% of control eyes[46]. Interestingly, this study indicated a decrease in inner and outer zone, central subfield thickness (CST) and reduction in total macula volume relative to control group[46].

Modern laser technologies and applications have been employed to treat DME. Such laser technologies include pattern scan laser photocoagulator (OptiMedica Corp, Santa Clara, CA) and NAVILAS (OD-OS Teltow, Inc. Germany). The laser beam delivery systems have short pulse duration that reduce heat thereby minimizes thermal damage at the site of application leading to patient compliance[47,48]. Other techniques such as subthreshold diode micro-pulse, navigated laser photocoagulation, pan retina photocoagulation and conventional single-spot laser application have been demonstrated to be more effective and safe to retina relative to conventional laser photocoagulation[47].

Although laser photocoagulation provides certain advantages, the associated drawbacks lessen enthusiasm and patient compliance. Drawbacks include destruction of photoreceptors due to laser photocoagulation, retinal scar formation and impedance of visual prognosis[49,50].

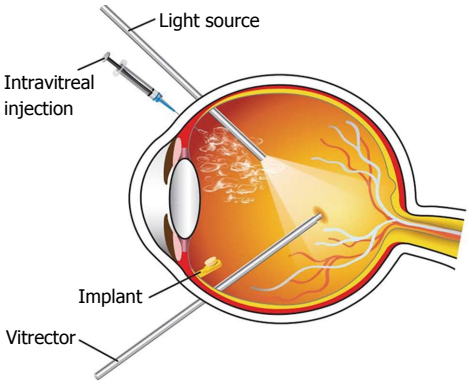

Figure 2 Treatment strategies of diabetic macular edema.

However, laser photocoagulation may be beneficial in DME subjects who do not respond to drug treatments[50]. Recently, a combination of intravitreal drug administration with laser photocoagulation have been investigated. Such treatment appears to be promising[45,51]. However, further studies may be required to establish the clinical benefit of the combination approach.

Vitrectomy

Vitreous plays an important role in the progression of DME. Studies demonstrated that improvement in vision for DME subjects may be achieved by induction of posterior vitreous detachment (PVD), pars plana vitrectomy (PPV), removal of internal limiting membrane (ILM) or taut posterior cortex[52-56]. However, the exact mechanism for vision restoration in DME with vitrectomy is yet to be delineated. Recent studies suggests that exclusion of vitreous gel may reduce the concentration of DME-promoting factors, alter vascular permeability and enhance retinal oxygen supply[11,57]. Vitrectomy may also improve vasoconstriction by lowering tissue pressure and elevating hydrostatic pressure gradient between the vascular and tissue compartments[18]. Moreover, vitrectomy improves vaso-permeability of the retinal endothelial cells and restore visual acuity. In a cohort study of vitrectomy in DME subjects, 87 eyes were evaluated visual acuity 20/63-20/400 including 54% ILM peeling, 61% epiretinal membrane peeling, and 40% panretinal photocoagulation[57]. Vitrectomy significantly reduced retinal thickness and improved visual acuity. However, vitrectomy is associated with side effects such as elevated intraocular pressure (IOP), vitreal hemorrhage, endophthalmitis, retinal detachment, induction of iris neovascularization and cataract formation[58]. Several randomized, controlled trials were conducted to investigate the side effects of vitrectomy on DME[59-65]. Such studies compared vitrectomy with laser, intravitreal steroid injection, and combinations. Vitrectomy may be applicable in DME subjects demonstrating epiretinal membrane and/or vitreomacular traction[66].

Intravitreal anti-VEGF therapy

Macromolecular therapy: VEGF plays an important role in retinal vascular permeability, breakdown of BRB and formation of macular edema. The current gold standard therapy for DME treatment is administering anti-VEGF agents[67-69]. VEGF inhibitors have demonstrated beneficial effects in DME treatment[70-74]. Current VEGF inhibitors include aflibercept (Eylea), RBZ (Lucentis), pegaptanib (Macugen), and bevacizumab (Avastin). RBZ and aflibercept are approved by Food and Drug Administration (FDA) for DME. Other anti-VEGF agents are also being considered due to cost effectiveness[75,76].

RBZ is a monoclonal antibody, approved for DME[77]. It has strong binding affinity to VEGF-A and blocks all isoforms of VEGF-A. Nguyen *et al*[78] demonstrated long term effects of RBZ in diabetic patients with DME. In this study, subjects were treated with RBZ, focal or grid laser or combination. The mean best-corrected visual acuity indicated that RBZ had significant effect to control edema in DME subjects. Moreover, a combination treatment with RBZ and focal/grid laser lowers edema residues also. Similarly, a clinical study, RIDE/RISE of RBZ demonstrated significant improvement in macula edema, accompanied by slow progress of vision loss in DME subjects[79-82].

Bevacizumab is full-length humanized monoclonal antibody with approximately three times larger molecular weight and size than RBZ. Bevacizumab also obtained FDA approval for the treatment of glioblastoma and colorectal cancer. However, it is being used as an "off-label" drug for DME treatment due to low cost. Several studies have reported bevacizumab to significantly improve macula edema and restore vision atleast partially in DME subjects[83-89]. Intravitreal injections of bevacizumab alone or in combinations with triamcinolone or photocoagulation were investigated. Interestingly, a combination of intravitreal bevacizumab and triamcinolone acetonide (TA) produced marginal improvement over bevacizumab alone in DME[90].

Aflibercept: (Eylea; Regeneron) *aka* VEGF Trap for eye is a soluble protein composed of binding domain for human VEGF receptor 1 (VEGFR1), 2 and Fc domain of human immunoglobulin G1[91,92]. Eylea has 100 times higher binding affinity to VEGF isoforms relative to bevacizumab or RBZ[93]. Moreover, aflibercept binds to special P1GF and VEGF-B and inhibits the activation of VEGFR1[93]. Korobelnik *et al*[91] conducted VISTA^{DME} and VIVID^{DME} phase three studies to compare the efficacy and safety of intravitreal aflibercept at 4 and 8 wk after initial monthly doses and laser treatment. Aflibercept demonstrated significant improvement in visual acuity over laser treatment. These results suggest that aflibercept is safe and well-tolerated. The most best corrected visual acuity (BCVA) can be achieved with aflibercept[94]. Many other studies such as VIBRANT, COPERNICUS, and GALILEO have reported significant benefits for aflibercept with better visual acuity[95-98]. Aflibercept did not cause significant difference at mild level of initial visual acuity relative to bevacizumab and RBZ. In fact, aflibercept can improve vision more effectively at worse level of initial

visual acuity[76,92].

Pegaptanib (Macugen) is a ribonucleic acid aptamer which was the first anti-VEGF approved by FDA for AMD. Macugen is another "off-label" drug for DME and has selective target to VEGF 165[99]. Several studies demonstrated pegaptanib to be safe, well-tolerated and superior efficacy in DME treatment[100-104].

Small molecule: Rapamycin or sirolimus is an immunosuppressive drug with anti-inflammation, antiangiogenic, antifibrotic, and antifungal properties. Sirolimus blocks interleukin-2-mediated signaling pathway and reduce VEGF production by inhibiting S6K1 phosphorylation[105-108]. Recently subconjunctival and intravitreal injections of sirolimus are applied for the treatment of DME, AMD and non-infectious uveitis patients which appear to be well tolerated[109-112]. Efficacy studies with sirolimus in DME subjects have also been conducted[109]. Moreover, aqueous nanomicellar topical drop of sirolimus has been developed. These nanomicellar constructs have been demonstrated to deliver sirolimus in high concentrations to back-of-the-eye tissues [retina/choroid] with topical drop[105].

Steroids and other treatments in DME

Inflammation plays a crucial role in DME pathogenesis. Though exact mechanism of corticoid action is unclear its use as anti-inflammatory agent is well recognized. It decreases VEGF activity and shows beneficial effects in DME[5,113-117]. Steroids may inhibit inflammatory cytokine production, leukostasis, and phosphorylation of cell-junction proteins[118].

TA is a synthetic steroid, recommended for DME treatment. TA displays anti-inflammatory and anti-angiogenic properties[119], improves tight-junctional levels between endothelial cells and reduces vascular leakage[120]. The widespread biological effects and large therapeutic window of intravitreal TA (IVTA) in the treatment of various ocular disorder is well known. It is prescribed as an "off-label" drug for DME and DR[121-124]. Several studies have been conducted to compare the safety and efficacy between IVTA and other treatments[67,90,125-128]. In a meta-analysis of randomized controlled trials study, IVTA demonstrated better vision acuity relative to standard care for ocular inflammation[126]. Moreover, IVTA administrations demonstrated short-term efficacy in retinal vein occlusion[129]. However intravitreal administration of IVTA, can also elevate IOP, accelerate cataract formation and cause other associated side effects such as endophthalmitis and pseudoendophthalmitis[130-134]. To overcome such side effects, recently aqueous nanomicellar topical drop of dexamethasone has been reported from our laboratory[135,136] that delivers therapeutic levels of the steroid to both anterior and posterior ocular tissues. Other studies for DME with corticoids include biodegradable dexamethasone implant (Ozurdex), surgically implantable reservoir of fluocinolone (Retisert), the dexamethasone intravitreal implant (Posidurex), and non-bioerodible injectable fluocinolone polymer (Iluvien)[137-143].

Emerging formulations for treatment of DME

Ophthalmic complications associated with diabetes are the leading cause of blindness in adults. In recent years, several formulations utilizing nanotechnology, anti-VEGF, VEGF trap, and implants for treating DME and other back-of-the-eye diseases are emerging. In addition, several combination therapies that involve two or more therapies together are being administered. Most of these drugs and combination therapies are either FDA approved or are in clinical trials and have shown tremendous improvement in vision to DME patients[78,144,145]. The following sections discuss different emerging formulations for treatment of DME.

CLINICAL STUDIES

Inhibition of VEGF has been indicated in AMD in recent years. Studies have shown that inhibition of VEGF can also be an effective interaction in the treatment and management of DME. Furthermore, intravitreal injection of anti-VEGF therapeutics (RBZ and aflibercept) was compared with laser monotherapy for treatment of DME on 1978 patients. Anti-VEGF therapeutics appeared to be statistically and clinically more superior to laser monotherapy[146]. Nguyen *et al*[82] conducted a phase Ⅲ randomized trial on 377 adult patients with vision loss due to DME. This study was conducted to evaluate efficacy and safety of RBZ administered at different dosages. Results indicated that after 24 mo of treatment 18.1% of sham patients gained more than 15 letters compared to 44.8% of patients treated with 0.3 mg of RBZ[82]. In addition RBZ showed rapid and sustainably improved vision with lower risk for further vision loss. This intervention significantly improved macular edema for DME patients[82].

Combination formulations are also emerging in the treatment of diseases associated with posterior segment of the eye. Combined regimens are utilized where retinopathies are not responding to one particular therapeutics strategy[147]. Liegl *et al*[51] conducted a study to evaluate a combination of laser photocoagulation and RBZ in the treatment of DME over one year period. One group receives combination therapy which involved 3 mo RBZ injections followed by laser photocoagulation. The second group is treated with RBZ injections only. BCVA is measured in both groups after treatment. An improvement in BCVA letter sore from 6.31 to 8.41 on both groups is observed. However, patients in monotherapy group require repeated RBZ injections (84%) relative to combined therapy (35%)[51]. These findings suggest that number of injections is significantly reduced with combination therapy. This may be beneficial to subjects since frequent intravitreal injections may result in local ocular complications such as endophthalmitis, retinal hemorrhage, retinal detachment and patient

noncompliance[148,149].

In addition to antibody therapeutics for treatment of DME, some promising strategies such as non-antibody drug products that have been used in the treatment and management of DME. Fluocinolone acetonide (FAc) (ILUVIEN®) was approved in 2014 by FDA for the treatment of DME. A long term follow-up study is conducted on DME subjects after receiving FAc intravitreal implant[150]. In this study subjects not responding to laser photocoagulation or anti-VEGF therapy were treated with FAc implant in one eye and anti-VEGF therapy in the contralateral eye[150]. Intravitreal FAc implant eye produced reduction in central macular thickness from 642 μm to 364 μm in the first month. On the contrary, eye treated with anti-VEGF therapy was unresponsive[150]. Similarly, another study that was conducted with FAc in chronic DME patients[151]. Results indicated an improvement of more than 15 letters on 34.0% patients treated with FAc compared to 13.4% on sham[151]. Such results provide an option for clinicians to treat subjects who do not respond to laser or anti-VEGF therapy. Moreover, FAc implant provides a long term sustained drug release of 0.2 μg/d for up to 3 years which can be more patient compliant therapy[150,151].

In this non-randomized, multicenter study, 2603 patients with macular edema and DME, Adelman *et al*[152] conducted a study to compare efficacy of anti-VEGF with triamcolone monotherapy and laser treatments. Despite the fact that all treatments revealed some improvement in visual acuity, anti-VEGF treatment showed the most improvement. However, treatment with PPV and ILM peeling exhibited improvement in vision acuity greater than anti-VEGF alone[152]. Consequently, this result indicates that treatment with ILM peeling and vitrectomy may be a better option to treat DME compared to other therapies.

Misra *et al*[153] have developed an insulin therapy that can be delivered to the retina. This is a sub-conjuctivally implantable hydrogel with thermosensitive and biodegradable properties for sustained delivery of insulin to the retina. Hydrogels are synthesized with UV photopolymerization of N-isopropylacrylamide monomer and dextran containing biodegradable oligolactate-(2-hydroxyetheyl methacrylate) units. Insulin loading efficiency was very high (98%)[153]. *In vitro* studies demonstrated that hydrogels were nontoxic when subjected to R28 retinal cells and can release active insulin for 7 d[153]. Such hydrogel implant may be utilized to load other macromolecular drugs intended to treat back-of-the-eye diseases.

Similarly, studies have been conducted to evaluate efficacy of combined treatments in DME. Vitrectomy combined with triamcinolone acetonide injection (IVTA) and macular laser photocoagulation was studied by Kim *et al*[147] for the treatment of non-tractional DME. This study was performed on 28 patients, who were sequentially subjected to vitrectomy, IVTA and macular laser photocoagulation. BCVA and CST were observed before vitrectomy, 1, 3, and 6 mo after the treatment. Results indicated substantial improvement in BCVA from 0.44 to 0.34 and from 433.3 to 310.1 for CST. These results suggest that combination of vitrectomy, IVTA and laser photocoagulation may be indicated in the treatment of DME.

Enzymatic vitrectomy for DME patients has recently been explored[154]. Diaz-Llopis *et al*[155] investigated the role of enzymatic vitrectomy through intravitreal injection of autologous plasmin enzyme in management of DME and DR. In a clinical study 63 eyes were treated with intravitreal injection of autologous plasmin enzyme and reexamined after one month for central macular thickness, BCVA and hyaloid. A second injection of this enzyme was administered to patients who did not develop PVD. Results indicated a massive improvement in central macular thickness by 100% and BCVA by 89%. However, PVD was observed to be 38% after first injection, which then improved to 51% after second injection[155]. Enzymatic vitrectomy is still new in the world of ophthalmology and further studies are required to understand the mechanism of action, efficacy and safety. Enzymatic vitrectomy may be considered as an alternative therapy for treatment of DME.

In a study with nine patients who had persistent DME, Zucchiatti *et al*[156] evaluated the effect of single injection of dexamethasone implant (0.7 mg) over 6 mo period. Results indicated a significant improvement in BCVA and central retina thickness which was sustained over 4 mo. A similar study was performed in DME patients with vitrectomized eyes for 26 wk by Boyer *et al*[139] to evaluate safety and efficacy of dexamethasone. A significant improvement in BCVA and central retinal thickness were maintained throughout the treatment period. In comparison, dexamethasone implant appeared to achieve superior outcomes in terms of BCVA, CMT with fewer injections compared to bevacizumab by Gillies *et al*[157]. Both treatments indicated excellent progress on vision impairment score. However, 11% of patients treated with dexamethasone implant lost 10 letters or more due to cataract formation[157]. FDA approved dexamethasone implant (Ozurdex) in the treatment of DME in 2014. This implant was previously approved for the treatment of non-infectious uveitis affecting posterior segment of the eye. Table 1 summarizes major clinical trials that have been performed to study biologics, steroid and implants in DME.

IN VITRO, IN VIVO AND PRE-CLINICAL STUDIES

As described earlier, DME is a back-of-the-eye disease. For local drug delivery, sub-conjunctival or intravitreal route of administration may be recommended. Since frequent injections are needed to maintain therapeutic levels, may cause complications such as retinal detachment, endophthalmitis, pseudoendophthalmitis and retina hemorrhage. Nanoparticle mediated sustained release formulations may lower frequent injections, and

Table 1 Major clinical trials performed to study biologics, steroids and implants for diabetic macular edema treatment

Trade name	Generic name	Study	Main conclusion	Ref.
Lucentis	Ranibizumab	RISE/RIDE	Ranibizumab improved vision and macular edema in DME patients	[82]
Eylea	Aflibercept	VISTA/VIVID	Intravitreal injection of aflibercept was shown to be superior compared to laser therapy in treatment of DME	[91]
Ozurdex	Dexamethasone implant	MEAD	Dexamethasone implant were well tolerated and improved BCVA in DME patients	[168]
Iluvien	Fluocinolone acetonide	FAME	Both low and high dose of Fluocinolone acetonide exhibited improved BCVA in treatment of DME	[169]

DME: Diabetic macular edema; BCVA: Best corrected visual acquit.

improve efficacy–leading to reduced side effects and improved patient compliance.

Recently, several groups have developed topical formulations for delivery to the retina. Cholkar *et al*[135] have reported a topical administration of mixed nanomicelle formulation (MNF) loaded with dexamethasone[136] rapamycin (sirolimus) and cyclosporine[158] for back-of-the-eye delivery[105]. MNF was found to be safe when tested on human retinal pigment epithelial cells (D407) and rabbit primary corneal epithelial cells *in vitro*. MNF can provide high drug loading and entrapment efficiency with an average size of 10.84 ± 0.11 nm. Furthermore, *in vivo* studies exhibited higher rapamycin concentration of 362.35 ± 56.17 ng/g in retina-choroid area but no drug was found in the lens or vitreous humor[105]. With these results, the topical administration may provide patient compliance since no injections are involved.

In addition, Fujisawa *et al*[159] have explored liposomal diclofenac eye drop formulation targeted to retina along with the aid of surface modification of liposomes. Liposomal formulation was prepared by using calcium acetate gradient method which increased entrapment efficiency from 51.3% (obtained by using hydration method) to 97%[159]. The researchers have utilized liposome surface modification with poly vinyl alcohol (PVA) or its derivatives (PVA-R) and observed that particle size of liposome with PVA modification to are 135 nm and 177 nm with PVA-R. *In vivo* studies performed on Japanese albino rabbits indicated an enhancement in accumulation of diclofenac in the retina-choroid by 1.8 fold with surface modified liposome relative to unmodified liposomes[159]. Higher entrapment efficiency may result in longer drug release. This delivery system may be suitable for the treatment of DME and other diseases associated with posterior segment of the eye.

RNA has been widely used as a therapeutic agent for treatment of wide variety of diseases. It involves modification, engineering, and/or assembly of organized materials at nanometer scale. The 117-nucleotide (nt) RNA, known as packaging RNA (pRNA) of bacteriophage and small interfering RNA (siRNA) have been widely applied in the treatment of cancer, viral infection, genetic diseases, and other diseases. Recently, Feng *et al*[160] have reported ocular delivery of pRNA (pRNA-3WJ and pRNA-X) nanoparticles and investigated distribution and clearance after subconjunctival injection. pRNA-3WJ and pRNA-X-nanoparticles labelled with Alexa647 and dsRNA were prepared and administered to mice by subconjunctival injection. It was observed that nanoparticles (pRNA-3WJ, pRNA-X and dsRNA) were distributed in corneal, sclera, and conjunctiva cells, but pRNA-X was found only in retina cells. This study suggests that RNA therapy for ocular diseases including back-of-the-eye delivery is feasible.

Similarly gene therapy for the treatment of inherited and acquired ocular diseases has been rapidly evolving in recent years. A major challenge for gene therapy is to overcome barriers associated with ocular gene delivery. This can be achieved by developing a suitable nanotechnology platform that can cross ocular barriers and deliver genes at target site. A polymer (natural or synthetic) or peptides have been employed to encapsulate DNA in polymer or peptide compacted DNA gene delivery nanoparticles[161]. Safety of compacted DNA nanoparticles for ocular delivery has also been investigated by Ding *et al*[162]. Polyethylene glycol substituted lysine peptide (CK30PEG) compacted DNA nanoparticles encapsulating EGFP vector were subretinally injected in mice at different dosages. Retina was observed at 1, 2, 4, 7 d post injection for any inflammatory signs or mediators. No inflammatory response was observed in the retina[162]. In addition, chitosan DNA nanoparticles for retinal gene delivery have been reported by Mitra *et al*[163]. Results indicate that compacted DNA nanoparticles may be exploited as gene therapies for treatment of the posterior diseases and particularly with RPE.

Carbon nanotubes are nanometer-scale tube-like cylindrical nanostructure. These cylindrical carbon molecules have unusual properties, which are valuable for nanotechnology, particularly in drug delivery. Nanotubes have also been explored for therapeutic delivery at back-of-the-eye. Panda *et al*[164] studied self-assembly dipeptide phenylalanine-α, β-hydrophenylalanine nanotubes for sustained intravitreal delivery of targeted tyrosine kinase inhibitor (pazopanib). The nanotube has a diameter and length of 15-30 nm and 1500 nm respectively. The nanotubes can be injected using 33G needle. Nanotubes loaded with a 25% w/w pazopanib were found to be nontoxic in *in vitro* studies. *In vivo* investigation was performed with pazopanib loaded nanotube for 15 d and the drug was observed in vitreous humor, retina and choroid RPE at 4.5, 5 and 2.5 times respectively compared to pazopanib solution[164].

These results suggest that nanotubes can be applied as a delivery system which may sustain higher drug concentration in ocular tissues.

Biodegradable polymers have been extensively utilized for the preparation of nanoparticles in drug delivery. Also nanoparticle in gel formulation of steroids has been reported for the treatment of macular edema by Boddu *et al*[165]. In this formulation PLGA (50:50 and 65:35) nanoparticles loaded with dexamethasone, hydrocortisone acetate, and prednisolone acetate were prepared by water in oil emulsion and then suspended in thermosensitive gel. Results indicated that entrapment efficiency for dexamethasone, hydrocortisone acetate and prednisolone acetate were 77.3%, 91.3% and 92.3% respectively. Drug release studies indicated no burst release and release kinetics followed zero order[165]. Nanoparticles suspended in thermosensitive gel may provide sustained release of drug at retina-choroid and may be exploited for DME and other ocular diseases.

A quench technology where nanoparticles in porous microparticles (NPinPMP) were prepared by superficial infusion and pressure for sustained bevacizumab delivery. The protein was coated with PLA nanoparticles and then mixed with PLGA microparticles. The particles were allowed to pass through supercritical carbon dioxide gas[166]. This allows expansion of PLGA matrix but not PLA matrix. Hence it creates porous PLGA microparticles in which encapsulated bevacizumab PLA nanoparticles are incorporated to generate NPinPMP. *In vitro* study indicated sustained release of bevacizumab for 4 mo with no change in conformation and activity[166]. Therefore, this formulation may be utilized with other protein therapeutics for the treatment of back-of-the-eye diseases and may reduce frequent injections to maintain therapeutic levels. However, size of microparticles may be controlled for intravitreal injections.

In addition, tailor made pentablock copolymer based formulation for sustained ocular delivery of protein therapeutics was extensively investigated by Patel *et al*[148,167]. Biodegradable pentablock copolymers (FDA approved) were synthesized by ring opening polymerization method using different monomers[148,167]. *In vitro* studies confirmed that polymers and monomers are safe and biocompatible when tested in ocular cell lines (APRE-19, SIRC, HCEC and RAW-264.7)[148,167]. Furthermore, pentablock nanoparticles loaded with FITC-BSA, IgG and bevacizumab were tested for particle size distribution which ranges between 320 and 355 nm. The entrapment efficiency, however, widely varied from 35% to 70%. *In vitro* studies indicate 40 d release of FITC-BSA and 60 d for IgG when nanoparticles are suspended in gel[167]. This IgG has similar molecular weight as bevacizumab, which can be delivered at the back-of-the-eye for the treatment of posterior diseases. Therefore, this formulation may be adopted to prepare other anti-VEGF therapies which can be delivered to the posterior ocular segment for DME and other retinal diseases.

DME is a disease associated with the posterior segment of the eye; therefore, it poses a significant delivery challenge. A significant portion of the drug may not reach back-of-the-eye due to associated barriers such as BRB, blood aqueous barrier, and vitreous barrier. Consequently only a small amount of drug reaches the back of eye. In order to maintain therapeutic drug levels, generally frequent intravitreal injections are required, which are not patient compliance and may cause other complications. In addition, delivery system that can sustain drug release for a prolonged period of time should be developed so that injection frequency can be minimized/avoided.

CONCLUSION

DME is a chronic disease leading to declined visual acuity and vision loss. It is a complex multifactorial disease which involves multiple pathways involving vision loss. At present, several novel drug delivery and treatment strategies have been developed to improve visual acuity and restore vision. The standard treatments of DME include laser photocoagulation, vitrectomy, intravitreal injections of anti-VEGF biologics and steroids. Because of destruction of photoreceptors due to laser photocoagulation, retinal scar formation and impedance of visual prognosis, it may be utilized in combination with vitrectomy or intravitreal injection. Moreover, the current understanding of DME pathophysiology has revealed a new therapy which includes targeted chemical mediators such as VEGF and inflammatory agents. The completion of several randomized, controlled trials in the long term may provide new therapeutics and novel delivery systems for the back-of-the-eye diseases.

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REVIEW

Non-selective beta-blockers in cirrhosis: Current concepts and controversies

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Abstract

Non-selective beta-blockers (NSBBs) have been at the forefront in the management of portal hypertension in liver cirrhosis for the last three decades, a trusty component in the armamentarium of the Hepatologist. The role of beta-blockers has been cemented for years in cardiac disease including angina, hypertension and in heart failure, however NSBBs with their non-selective effects on β1 and β2 receptors have led to them fondly being termed "the hepatologist's aspirin". NSBBs' role in reduction of portal pressure in the setting of primary and secondary prophylaxis for variceal haemorrhage has been well established. NSBBs include propranolol, nadolol and carvedilol - with the latter having been shown to be effective in patients who often fail to demonstrate a haemodynamic response to propranolol. Recent observational studies however have served for the Hepatology community to question the beneficial role of NSBBs in portal hypertension, especially in advanced cases with refractory ascites. The deleterious effect in patients with refractory ascites in a few studies led to a U-turn in clinical practice, with some in the Hepatology community withdrawing their usage in patients with advanced cirrhosis. This also led to the "window hypothesis" suggesting there may be only be a finite time frame when NSBBs have a beneficial effect in portal hypertension. The window hypothesis proposed the window for the benefits of NSBBs is closed in early portal hypertension, opening as portal hypertension progresses with it closing in advanced liver disease. The window was proposed to close in conditions such as refractory ascites or spontaneous bacterial peritonitis when patients may not necessarily mount a compensatory haemodynamic response when on NSBBs. Some centres however have continued the practice of NSBBs in advanced cirrhosis with published data challenging the scepticisms of other groups who stop NSBBs. Thus the debate, like the window hypothesis has opened, with more questions to be answered about NSBB's mechanism of action not only in reducing portal hypertension but also their effects on systemic haemodynamics and on the pro-inflammatory pathways often activated in cirrhosis especially in advanced disease. This article serves to review the role of NSBBs in the management of portal hypertension/cirrhosis and concentrate on current concepts and controversies in this field.

Key words: Variceal haemorrhage; Non-selective beta-

blockers; Portal hypertension; Liver cirrhosis

Core tip: This article serves to discuss the changing role of non-selective beta-blockers in liver disease and portal hypertension. For many years non-selective beta-blockers have been at the forefront in reducing portal hypertensive complications such as variceal haemorrhage, however recent data has questioned their role in advanced liver disease. This article reviews their role in portal hypertension, discusses recent advances in the field and reviews the controversy recently generated regarding their role in advanced liver disease.

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INTRODUCTION

Liver cirrhosis is a major cause of morbidity and mortality throughout the world[1,2], with the advent of portal hypertension one of the key defining steps leading to the complications that can develop in advanced liver disease. The role of non-selective beta-receptor antagonists [non-selective beta-blockers (NSBBs)] has been well established over the years in the Hepatologist's armamentarium against portal hypertension and its consequences. NSBBs have been used routinely in practice with beneficial roles in primary and secondary prophylaxis in patients with medium to large oesophageal varices[3-5]. The development of portal hypertension is the key defining step leading to complications that can occur in patients with liver cirrhosis irrespective of aetiology. Consequences can include variceal haemorrhage, ascites formation [and thereafter a risk of the development of spontaneous bacterial peritonitis (SBP)] and hepatic encephalopathy. Portal hypertension develops from a combination of a rise in the intrahepatic resistance but also from splanchnic vasodilatation and the hyperdynamic circulation that occurs in cirrhosis. It has been shown that rupture of varices is related to tension on the variceal wall, with the tension dependent on the radius[6]. There has been no linear relationship found between the severity of portal hypertension and the risk of variceal haemorrhage however a hepatic venous pressure gradient (HVPG) > 12 mmHg has become an accepted threshold for variceal bleeding[7,8]. As liver disease progresses and portal hypertension worsens, ascites can form, bacterial translocation from the gut occurs and patients can become prone to complications such as infection that can in turn lead to increases in portal pressure and thus variceal haemorrhage. Thus reduction of portal pressure is key in preventing complications of cirrhosis including reduction of ascites, hepatic encephalopathy and variceal haemorrhage[9-11]. Whilst portal hypertension can be reduced by radiological methods such as transjugular intrahepatic portosystemic shunts (TIPSS), NSBBs have been the key pharmacological therapy in reduction of portal pressure over the years. The role of selective beta-blockers in cardiac disease has been cemented for years, including in acute coronary syndrome[12], hypertension[13] and congestive cardiac failure[14]. NSBBs used in liver disease however act by dual blockage of β1 and β2 receptors unlike their cardio-selective counterparts. NSBBs reduce cardiac output (CO) and splanchnic blood flow *via* blockade of the β1 receptor, and by blocking β2 result in a splanchnic vasoconstriction *via* unopposed α1 activity[15]. NSBBs have been used to decrease the incidence of 1st variceal haemorrhage in patients with cirrhosis (*i.e.,* primary prophylaxis)[3-5] and then to prevent rebleeding after a variceal haemorrhage (*i.e.,* secondary prophylaxis)[16-18]. Propranolol has been the mainstay of NSBBs used in chronic liver disease for years, however more recently carvedilol, with its intrinsic α1-adrenergic activity has been studied and found to be useful even in settings where patients have failed to demonstrate an appropriate haemodynamic response to propranolol[19] thus providing an additional or alternative therapy for reduction of portal pressure.

There has however been a recent concern about the role of NSBBs in advanced liver disease and especially in patients with refractory ascites, with one group raising concerns showing an increase in mortality in this setting[20]. This issue led to the "window" hypothesis, suggesting that there may be a finite time frame when NSBBs have a favourable effect in chronic liver disease, with their effects becoming deleterious and the window closing in advanced disease states[21]. However, recent data from our own centre has argued against this, with beneficial findings of NSBBs in patients with ascites on a liver transplant waiting list even in those patients with refractory ascites[22]. Furthermore, favourable data on the role of NSBBs in alcoholic hepatitis[23] and acute on chronic liver failure (ACLF) has recently emerged[24]. Thus, there still remains controversy of how safe and effective are NSBBs in advanced cirrhosis, with further studies required to address this debate.

A PubMed search was performed using the following keywords: "non-selective beta-blockers" and "variceal haemorrhage cirrhosis". From this search 2965 articles were found. This search was complemented by a search of the keywords using www.google.com™. One hundred and eighteen papers/abstracts were studied for the preparation and writing of this review article. This review article serves to explore the role of NSBB in portal hypertension and liver cirrhosis, to review their mechanism of action and to review the favourable and negative data pertaining to their roles in liver disease. The article will review the recent controversies with NSBB in advanced liver disease, and proposes some thoughts on future directions of NSBB usage and studies potentially required to answer the question if NSBBs can remain as the Hepatologist's aspirin?

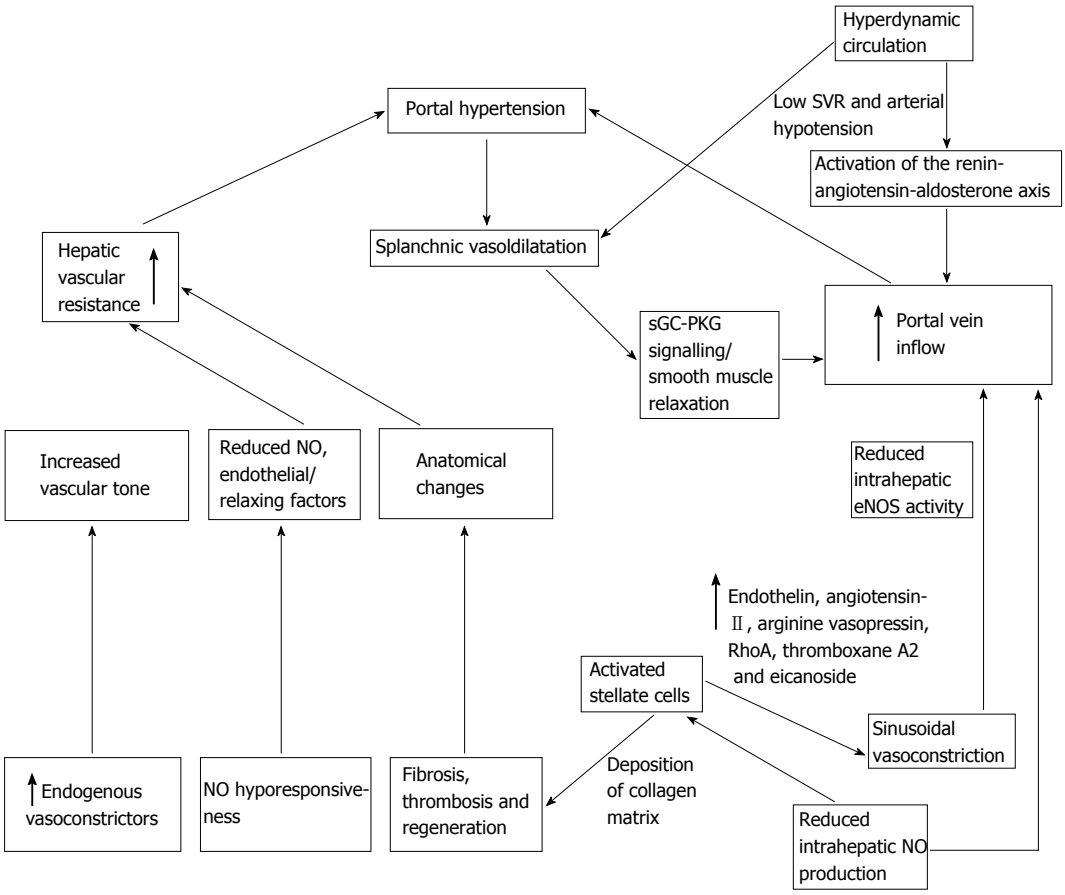

Figure 1 Factors involved in the pathogenesis of portal hypertension. A mechanical obstruction due to fibrosis or regenerative nodules results increased resistance to flow and a rise in hepatic vascular resistance. Contraction of sinusoidal and extra sinusoidal contractile cells (stellate cells) with intrahepatic imbalance between vasoconstrictors (such as endothelin-1 and angiotensin) and vasodilators (*e.g.*, NO) leads to reduced intrahepatic eNOS activity leading to an increase resistance to portal inflow. Portosystemic collaterals develop with the aim of decompressing the portal circulation. However, the opposite occurs, with splanchnic vasodilatation in response to a relatively ischaemic liver or extrahepatic excess of NO, with sGC-PKG signalling and smooth muscle cell relaxation. The increased portal blood flow maintains portal hypertension. A hyperdynamic circulation results due to these haemodynamic changes in cirrhosis and portal hypertension. eNOS: Endothelial nitric oxide synthase; NO: Nitric oxide; SVR: Systemic vascular resistance; sGC-PKG: Soluble guanylyl cyclase-cGMP-dependent protein kinase.

PORTAL HYPERTENSION: THE KEY TARGET FOR NSBBS

The development of portal hypertension is the key factor leading to decompensation of liver disease such as variceal haemorrhage, ascites formation (with the inherent risk of development of SBP thereafter) and also hepatic encephalopathy[25]. Portal hypertension results from 2 major events: An increase in the intra-hepatic vascular resistance and also an increase on portal venous inflow (Figure 1). Increased intrahepatic resistance can result of a number a pathophysiological mechanisms occurring in liver disease. Structural fixed anatomical changes can result in up to 70% of the cause of intrahepatic resistance increasing[26] with the suggestion that the other 30% the result of an increase in vascular tone. Anatomical disruption within the liver develops by the activation of stellate cells, which in turn, leads to sinusoidal capillirisation. Stellate cells are found in the perisinusoidal space (Space of Disse) within the liver. These cells are the major cell subset involved in liver fibrosis. In health, stellate cells are in an inactivated quiescent state, however when the liver is injured the stellate cells become activated and secrete collagen scar tissue. A reduction in the nitric oxide (NO) production by sinusoidal endothelial cells furthermore causes activation of stellate cells. The stellate cells once activated are the key mediator in the extracellular matrix production[27]. Over time, the formation of fibrous septae and also nodular regeneration leads to alteration of the hepatic architecture, and micro-portal and hepatic venule thrombosis also leads to an increase in the intra-hepatic resistance[28]. All these factors lead to a fixed component of the rise in portal pressure. It is clear that anatomical changes at present are a fixed component in the development of portal hypertension, abrogated by either replacement of full liver tissue (*e.g.,* by liver transplantation), or by bypassing the fixed anatomical restriction (*e.g.,* by a TIPSS). Research strategies are ongoing to modulate the scarring/fibrosis pathways to try to address this "fixed" component at present of portal hypertension[29].

The other more dynamic component to the development of portal hypertension is the change in the vascular tone and portal inflow increase. In cirrhosis activated stellate cells cause sinusoidal vasoconstriction due to an increase in

Table 1 Changes in measurement of portal haemodynamic pressures with different types of portal hypertension

Type of portal hypertension	Example	FHVP	WHVP	HVPG
Pre-hepatic	Portal/splenic vein thrombosis	Normal	Normal	Normal
Pre-sinusoidal	Primary biliary cirrhosis, schistosomiasis, sarcoidosis	Normal	Normal	Normal
Sinusoidal	Alcoholic hepatitis, NASH/alcoholic/viral cirrhosis	Normal	Increased	Increased
Post-sinusoidal	Sinusoidal obstruction syndrome	Normal	Increased	Increased
Post-hepatic	Budd Chiari[1]	-	-	-
	Heart failure	Increased	Increased	Normal

[1]Denotes hepatic vein not cannulated to measure. NASH: Non-alcoholic steatohepatitis; HVPG: Hepatic venous pressure gradient; FHVP: Free hepatic vein pressure; WHVP: Wedged hepatic vein pressure.

vasoconstrictive mediators such as endothelin, angiotensin-Ⅱ, arginine vasopressin, RhoA, thromboxane A2 and eicanoside[30-33]. There is also decrease in the vasodilators at a sinusoidal level such as NO - a key vasodilatory mediator in the portal venous system[34] and glucagon. Thus an imbalance exists leading to a reduction of intrahepatic endothelial Nitric Oxide synthase activity, leading to an increase in portal inflow. This mechanism is modifiable *via* NSBBs as is the other major component in the development of portal hypertension - the increased portal inflow (Figure 1).

Ohms law states that the change in pressure (P1-P2) along a blood vessel is a function of the resistance (R) and the rate of blood flow (Q), expressed as P1-P2 = R × Q. In healthy individuals, the liver accommodates changes in blood flow throughout the day and the liver itself is a very low resistance organ. The liver accommodates changes in blood flow without increasing the portal pressure by reducing the pressure in the liver by the recruitment of additional hepatic sinusoids. Thus (P1-P2) does not increase as Q increases but R falls. When Ohms law is applied in advancing liver disease, there is an increase in (intra-hepatic) resistance (R) leading to a rise in pressure (P1-P2), and the formation of porto-systemic collaterals to decompress the higher pressure. Also due to a hyperdynamic circulation there can be an increase in Q, again leading to an increase in (P1-P2) in Ohms law.

Splanchnic vasodilatation occurs as a response to a relatively ischaemic liver or due to extrahepatic excess of NO. This results in soluble guanylyl cyclase-cGMP - dependent protein kinase signalling and smooth muscle cell relaxation[35]. The resultant increased portal blood flow maintains portal hypertension and the hyperdynamic circulation results due to these haemodynamic changes in advancing liver disease. This manifests as a high CO with low systematic vascular resistance (SVR) and arterial hypotension[36]. The hyperdynamic splanchnic circulation (leading to increased portal inflow) is thus one of the key factors involved in the maintenance of portal hypertension and an area where NSBB have a key mechanism of action. The circulatory disturbances that arise (including reduction in CO, increase in heart rate and decrease in mean arterial pressure (MAP) and a reduction in the SVR) can lead to activation of the sympathetic nervous system and also the renin-angiotensin system in an attempt to counteract low arterial pressure[37]. There is an increase thus in not only sodium retention but also total body water retention (often leading to a dilutional hyponatraemia) and plasma/blood volume. Despite this, patients with cirrhosis and advanced disease have a reduced effective arterial blood volume[38,39] which can lead to organ hypoperfusion and problems with hepatorenal syndrome (HRS), or when infection takes hold with further arterial vasodilatation. Also patients can thus encounter problems with paracentesis-induced circulatory dysfunction (PICD) when ascitic fluid is removed without adequate plasma expansion replacement. With this pathophysiology, it is here that NSBBs indeed may have a beneficial effect in portal hypertension but also may worsen advanced systemic haemodynamic changes in end stage disease[40].

Markers for NSBB effectiveness not only include clinical endpoints such as prevention of variceal haemorrhage or rebleeding from varices, but reductions of portal pressure. Portal pressure measurements can be directly derived from the HVPG (normal range 1-5 mmHg). The HVPG can be measured by advancing a catheter either by a transfemoral or transjugular route into the hepatic vein and here a free hepatic vein pressure (FHVP) is measured. A balloon is then used to wedge the catheter in the hepatic vein and a second pressure is taken [the wedged hepatic vein pressure (WHVP)]. The WHVP reflects sinusoidal pressure. Thereafter the HVPG can be calculated (HVPG = WHPG - FHVP)[41]. The differences between these components depending on the type of portal hypertension are summarised in Table 1. Varices have been shown to be more likely to develop when the HVPG > 10 mmHg[42]. To effectively reduce the risk of variceal haemorrhage, the drop in portal pressure (as measured by the HVPG) must be reduced to < 12 mmHg or by 20%[43] thus allowing a surrogate marker for NSBB effectiveness in any clinical trial or occasionally in clinical practice when indicated.

NSBB - MECHANISM OF ACTION AND FAVOURABLE EFFECTS IN CHRONIC LIVER DISEASE

NSBB: Mechanism of action

NSBBs and their use in liver disease stems back over 3 decades[3] with a well understood mechanism of action

in reducing portal hypertension. NSBBs effects are not only *via* a β1-receptor to reduce the cardiac output and splanchnic blood flow[44,45] but also an additional action *via* β2 receptor blockade, blocking the adrenergic dilatory tone in mesenteric arterioles, thus resulting in an unopposed α-adrenergic vasoconstriction and subsequent reduction in portal blood flow. This "β-2" effect occurs after chronic usage[4]. This dual action is very much different to their counterparts used in cardiac disease such as metoprolol or atenolol that have been shown to be less effective than the NSBBs and thus not recommended in portal hypertension[46,47]. NSBBs include propranolol, nadolol and carvedilol. Propranolol has been used since the original study by Lebrec *et al*[48] in the 1980s however its effect of HVPG reduction can be variable with up to 31% reduction seen in some studies[15]. Up to a third of patients however do not have an adequate haemodynamic response to propranolol despite reductions in azygous flow[49]. Nadolol is another NSBB used with a longer half-life than propranolol due to low lipid solubility and hepatic metabolism[50] which allows for a once-a-day regime as appose to the twice a day of propranolol. It has a similar haemodynamic effect as propranolol[51]. Timolol is another NSBB like nadolol with low lipid solubility, and a greater affinity for β-1 and β-2 receptors[50]. However there is a paucity of comparative data in the setting of this drug in portal hypertension[52].

Carvedilol is one of the new generations of NSBBs with promising data in the setting of portal hypertension. It has an additional vasodilating action due to unopposed alpha-1-receptor blockade. This additional blockade results in a reduction of portocollateral resistance, and a reduction of intrahepatic resistance *via* an effect on hepatic stellate cells[15]. It has been found to be 2-4 times more potent action compared to other NSBBs at a receptor blockade[15]. Carvedilol is protein bound thus in patients with cirrhosis and hypoalbuminaemia there can be an increased bioavailability of it. It has anti-inflammatory, anti-oxidant[53] properties and also an antifibrotic effect[54] along with other roles in enhancing insulin sensitivity and improving mitochondrial function[55]. The role of carvedilol in reducing portal pressure has been compared to other NSBBs. It has been found after chronic usage to reduce HVPG[56] with more patients having a haemodynamic response when compared to propranolol[57]. In another study from Reiberger *et al*[19] the benefits of carvedilol were established in those not responding to propranolol. In this study, 56% of patients who did not respond to propranolol showed a haemodynamic response to carvedilol. There was a drop in HVPG of -19% in the carvedilol group *vs* -12% in the propranolol group. Thus there may indeed be a subset of patients deemed propranolol "haemodynamic non-responders" who are at risk of bleeding despite being on NSBB. Further studies are thus required to assess if carvedilol should be used as first line, or whether all patients on propranolol should have (ideally non-invasive) an assessment for haemodynamic response in the clinical setting and then switched to carvedilol if indeed a "non-responder". Carvedilol has also been compared to variceal band ligation (VBL) in the prevention of 1st variceal haemorrhage with medium or large varices[58]. The NSBB group had lower rates of 1st variceal bleed of 10% *vs* 23% in the band ligation group on intention-to-treat analysis, although there was no difference in mortality or bleeding related mortality between the groups. In this study carvedilol was well tolerated[58] at dose of 12.5 mg and higher doses have shown to have no additive effect in reduction of portal pressure from this dose[19].

Another area of benefit of NSBBs in patients with portal hypertension may indeed include reducing in bacterial translocation. In mice models, propranolol treated mice have been shown to not only have significantly lower portal pressures, but faster intestinal transit times and also lower rates of bacterial overgrowth and translocation[59]. In a meta-analysis by Senzolo *et al*[60], 644 patients (257 propranolol-treated) were evaluated (73% with ascites). The end-point of advent of SBP was used in the 3 randomised controlled trials (RCTs) and 1 primary prophylaxis study, and a statistically significant difference of 12.1% ($P < 0.001$) in favour of propranolol in preventing SBP was found. The beneficial effects were found irrespective of fall of portal pressure measurements thus suggesting an independent effect of NSBB in prevention of SBP irrespective of their benefits in reduction of portal pressure. Reiberger *et al*[61] have recently shown that NSBB therapy decreases intestinal permeability and plasma LPS-binding protein (LBP - a soluble acute phase response protein) and interleukin-(IL-)6 (a pro-inflammatory cytokine related to fever generation and related to such conditions as Systemic Lupus Erythymatosis and Rheumatoid arthritis[62,63]) with higher levels of IL-6/LBP associated with a higher risk of variceal bleeding on follow-up but not mortality. Thus NSBBs have a number of different mechanisms whereby they may indeed have benefit in patients with portal hypertension especially when varices develop (Table 2).

Clinical indications: Oesophageal varices

In the seminal Phase-Ⅲ study of NSBBs in patients with oesophageal varices by Lebrec *et al*[3] 74 patients who had a variceal 1st (index) bleed were randomised to either treatment with propranolol orally or placebo with 96% of the NSBB group free from bleeding at 1 year compared to 50% patients in the placebo group ($P \leq 0.0001$). The role of NSBBs in prevention of 1st variceal haemorrhage (*i.e.,* primary prophylaxis) was studied with Pascal *et al*[4] randomising 230 patients with large oesophageal varices (with no history of previous bleeding) to propranolol or placebo and finding 74% *vs* 39% free from bleeding at 1 year respectively ($P < 0.05$). There was also a survival advantage in the NSBB arm (72% *vs* 51% placebo, $P < 0.05$) thus showing a definite role in primary prevention, echoed thereafter in a number of studies including other NSBBs such as nadolol[64,65]. A meta-analysis of 9 placebo-controlled trials (964 patients) found the -11% (95%CI: -21% to -1%) for bleeding and -9% (95%CI: -18% to -1%) for death when propranolol was

Table 2 Types of non-selective beta-blocker used in cirrhosis

	Propanolol	Carvedilol	Nadalol
Proposed mechanism of action	β-1 activity to reduce cardiac output and reduce portal blood flow through splanchnic vasoconstriction *via* β-2 blockade	β-1 activity to reduce cardiac output and reduce portal blood flow through splanchnic vasoconstriction *via* β-2 blockade. Additional intrinsic α1-adrenergic activity	β-1 activity to reduce cardiac output and reduce portal blood flow through splanchnic vasoconstriction *via* β-2 blockade
Side efffects/cautions[1]	Hypotension, bradycardia, caution in peripheral vascular disease/asthma. To be discontinued at time of SBP, renal impairment and hypotension[1]	Hypotension (more profound than others), bradycardia, caution in peripheral vascular disease/asthma. To be discontinued at time of SBP, renal impairment and hypotension[1]	Hypotension, bradycardia, caution in peripheral vascular disease/asthma. To be discontinued at time of SBP, renal impairment and hypotension[1]
Indications	Primary prophylaxis of variceal haemorrhage (Level 1A, grade A). In combination with VBL for secondary prevention (Level 1a, grade A)[2]	Primary prophylaxis of variceal haemorrhage (Level 1a, grade A). In combination with VBL for secondary prevention (Level 1b, grade B)[2]	Primary prophylaxis of variceal haemorrhage (Level 1a, grade A). In combination with VBL for secondary prevention (Level 1a, grade A)[2]
Dose	40 mg BD if tolerated or once HR < 50-55 bpm	12.5 mg OD if tolerated or once HR < 50-55 bpm	40mg OD (maximum dose 240 mg) or once HR < 50-55 bpm
Mode of administration	Oral	Oral	Oral

[1]For consideration of re-introduction after acute event and depending on clinical judgement; [2]NSBB combined with VBL as standard of care in secondary prevention[23,65]. BD: Bi-daily; OD: Once daily; SBP: Spontaneous bacterial peritonitis; VBL: Variceal band ligation; NSBB: Non-selective beta-blocker.

compared to placebo[66]. Primary prophylaxis of variceal haemorrhage with a NSBB is thus recommended at present in patients with medium/large varices[25,67].

The role of NSBBs in primary prophylaxis in patients with no varices or small varices has also been studied. In a RCT patients with small varices were studied and in the group receiving NSBB actually developed more varices in the NSBB arm[68]. In another trial[69], nadolol reduced the incidence of variceal bleeding when compared to placebo but had no survival benefit and more side effects. In another study of NSBBs in an unselected group of patients with chronic liver disease (without cirrhosis or varices in some patients) no benefit was found in the use of NSBBs[70] in prevention of 1st variceal bleed or survival. Another RCT looked at the role of Timolol *vs* placebo in patients without varices but portal hypertension (HVPG > 6 mmHg) and found no difference in variceal bleeding rates, and in fact more side effects of patients on NSBBs[42]. In a meta-analysis of 6 RCTs of cirrhotic patients with small or no varices, incidence of large varices, 1st upper gastrointestinal bleed and death were similar between placebo and NSBB groups[71]. Thus NSBB are not currently recommended for primary prophylaxis in patients without endoscopic evidence of varices or small varices[25,67]. Combination of NSBB and Isosorbide Mononitrate (ISMN - another vasodilator used in patients with angina) has been studied in the setting of primary prophylaxis for variceal bleeding. Nadolol alone *vs* nadolol and ISMN was studied in a RCT, with combination therapy leading to reduced frequency of bleeding however no significant differences in mortality[72]. These findings were not echoed however in a double-blind RCT comparing propranolol and ISMN *vs* propranolol and placebo[73]. With a potential for increased side effects due to hypotension, this strategy is thus currently not recommended for primary prophylaxis[67].

The role of NSBBs in secondary prevention of variceal haemorrhage after an index (1st) variceal bleed has become established over the years[74-76]. A meta-analysis of NSBB (nadolol or propranolol in 12 trials) compared to no treatment found a significant reduction in rebleeding but no mortality benefit[77,78]. The addition of ISMN in a secondary prophylaxis role has shown improved varcieal rebleeding rates in one study[79], but no survival benefit. In a meta-analysis of ISMN alone *vs* ISMN with NSBB or endoscopic therapy showed there was no mortality benefit from combination of ISMN/NSBB than NSBB monotherapy[80]. There have been a number of studies and meta-analysis comparing combined endoscopic VBL and NSBB and monotherapy with either. A large meta-analysis of 23 trials of either VBL or injection sclerotherapy in combination with NSBB concluded that combination therapy led to reduced rebleeding than either NSBB alone or endoscopic therapy alone, however no difference in mortality was found[81]. There has been a number of meta-analysis of numerous trials since then with differing results on combination therapy affecting mortality but a clear benefit in reduction of rebleeding[17,82-84]. A recent multicentre RCT from Stanley *et al*[85] compared carvedilol to VBL in rebleeding and although found a tendency towards improved survival in the carvedilol arm, there was no statistically significant difference in rebleeding rates or mortality (P = 0.857 and P = 0.110, respectively). Combination therapy of VBL and NSBB (propranolol or nadolol) is however now recommended for prevention of variceal rebleeding[25,67].

NSBBs clinical indications: Gastric varices

NSBBs have also been studied in the setting of gastric varices, which historically are known to bleed at a lower portal pressure than their oesophageal counterparts with poorer outcomes[86]. Mishra *et al*[87] studied the role of NSBB (*vs* glue therapy) in primary prophylaxis, comparing them to injection therapy with N-Butyl-2-

Cyanoacrylate glue therapy in the prevention of rebleeding of gastric varices. In 67 patients, the group receiving injection therapy after index bleed had a lower rebleeding rate and lower mortality when compared the NSBB group (15% *vs* 55%, $P = 0.004$ and 3% *vs* 25%, $P = 0.024$ respectively). In another paper in the setting of gastric varices, Hung *et al*[88] compared the effects of endoscopic injection obturation therapy alone compared to that of obturation combined with NSBB in 95 patients after a gastric variceal haemorrhage. Overall rebleeding and survival rates were not different between the two groups ($P = 0.336$ and 0.936, respectively), thus the optimal role of NSBB in patients after an index gastric variceal haemorrhage remains in question. The British Society of Gastroenterology (BSG) recent guidelines[67] stated that NSBB treatment could be considered in selected high risk patients with large gastro-oesophageal type 2[86] (extending down from the oesophagus below the gastro-oesophageal junction into the fundus) after "taking into account the patient's preferences and clinical judgment".

NSBB: THE CURRENT CONTROVERSIES

NSBBs: A deleterious role in advanced cirrhosis?

With the role of NSBBs in portal hypertension and variceal haemorrhage prevention established, the tide however has changed in the last few years with a series of high profile publications questioning their safety in advanced cirrhosis[20,21,89,90]. The detrimental effect of NSBB in patients with ascites was initially provoked in a study by Bañares *et al*[57] with the aim of the study to explore the role of NSBBs in reducing HVPG when patients randomized to carvedilol or propranolol, with the former showing a greater reduction in HVPG (19% ± 2% *vs* -12% ± 2%, $P = 0.001$). There was however a tendency towards an increase in the dose of diuretics required in the carvedilol arm (27% *vs* 8%, $P = 0.07$), suggesting that carvedilol may worsen ascites. As cirrhosis progresses after the development of varices, ascites later can form as the disease gets more advanced as proposed by D'Amico *et al*[75]. Thus with a suggestion of ascites being worsened by NSBB in the study by Bañares *et al*[57], the role of NSBBs in advancing cirrhosis was studied in more detail.

The potential detrimental effect of NSBB in the setting of patients with ascites undergoing a large volume paracentesis (LVP) was studied by Sersté *et al*[89]. In this cross over trial of 10 patients, haemodynamics and plasma renin levels were assessed pre-, immediately post- and 7 d post- LVP in patients on propranolol. The NSBB was phased out and then measurements repeated in a similar fashion. When on NSBB immediately post-LVP the HR did not change ($P = 0.61$) however the MAP significantly fell ($P = 0.007$). When off NSBB, immediately post-LVP the MAP significantly fell again ($P = 0.003$) however with a significant rise in HR ($P = 0.001$). The authors proposed that immediately post-LVP that NSBB may indeed cause a PICD with a lack of rise of compensatory HR in patients on NSBB, which may account for a degree of tissue hypoperfusion. Thus it was proposed that NSBB may indeed contribute to PICD in patients on NSBBs. It is however worth noting that these findings were not replicated in another study[91] exploring the relationship between changes in HVPG induced by NSBB and the development of ascites in compensated cirrhosis (with severe portal hypertension). Eighty-three patients without any previous decompensation of cirrhosis, HVPG ≥ 12 mmHg and large oesophageal varices were included. Haemodynamic studies prior to NSBB (nadolol) were performed and then repeated at 1-3 mo later. This group showed that patients in whom NSBB reduced HVPG by ≥ 10% ("NSBB-responders") indeed had a lower probability of developing ascites (19% *vs* 57% at 3 years, $P < 0.001$), refractory ascites ($P = 0.007$), and HRS ($P = 0.027$). It is worth noting however that these two studies[90,91] were not directly comparable due to slightly different patient cohorts in that one had decompensated patients and the other compensated cirrhotic patients.

The role of NSBBs in refractory ascites has further been questioned by the same French group in a high impact publication[20] out-with the paracentesis setting. In this prospective landmark study, 151 patients were studied (77 on propranolol) with refractory ascites. The 1-year survival was indeed worse than those receiving propranolol [19% (95%CI: 9%-29%) *vs* 64% (95%CI: 52%-76%), $P < 0.0001$)]. Along with NSBB, hyponatraemia, Childs C class and renal dysfunction were predictors of mortality on multivariant analysis. It was concluded that NSBBs are contraindicated in patients with refractory ascites and led to a change in the use of NSBBs in cirrhosis in some parts of the international Hepatology community.

In advanced cirrhosis when bacterial translocation is high, and patients are prone to infections, the role of prophylactic antibiotics is clear but the place of NSBBs has been cast into some doubt. Mandorfer *et al*[90] explored in a retrospective cohort of 607 patients the effects of NSBBs in advanced cirrhosis. NSBBs were shown to improve transplant-free-survival in patients without SBP - HR = 0.75; 95%CI: 0.581-0.968; $P = 0.027$). On development of SBP however, NSBBs were associated with haemodynamic compromise (systolic arterial pressure < 100 mmHg 38% *vs* 18% those not on NSBB, $P = 0.002$), but more importantly increased incidence of HRS (24% *vs* 11%, $P = 0.027$), and reduced transplant free survival (HR = 1.58, 95%CI: 1.098-2.274, $P = 0.014$). This along with the data from a meta-analysis by Senzolo *et al*[60] showing NSBB preventing SBP potentially suggests NSBBs are indeed beneficial in prevention of SBP until late on when infection sets in and patients have difficulty mounting a compensatory cardiac/organ perfusion response on NSBBs.

NSBBs and the window hypothesis

Following on from the Sersté *et al*[20,89] studies, it was proposed that NSBBs were only beneficial during a set time window in the progression of cirrhosis with portal hypertension[21]. The "window hypothesis" proposed

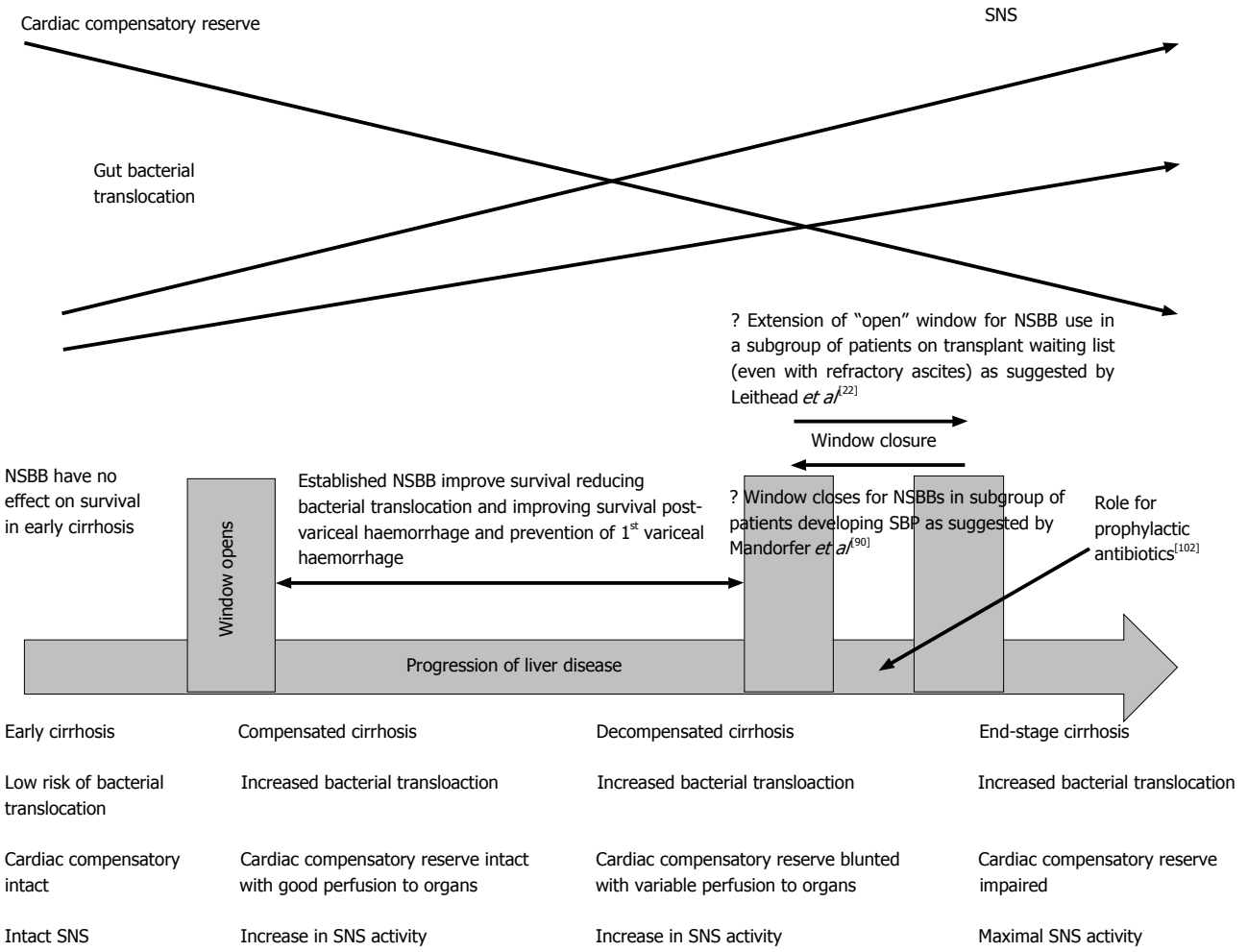

Figure 2 Extended - "window hypothesis" adapted and revised from Krag *et al*[21]. The window hypothesis illustrates that in early portal hypertension when low risk of bacterial translocation and adequate cardiac compensatory reserve, NSBBs have no effect on survival[25,67]. As disease progresses and varices enlarge there is clear benefit of NSBB in primary and secondary prophylaxis in improving mortality and also reducing rebleeding rates. The original widow hypothesis[21] commented that the window for benefit of NSBBs then may indeed close in decompensated cirrhosis (*e.g.*, patients with refractory ascites[20]) however the data from Leithead *et al*[22] would suggest the window may indeed remain open even in such patients for a period of time. This window however may indeed close once patients have developed an episode of SBP[90]. SNS: Sympathetic nervous system activity; NSBB: Non-selective beta-blocker; SBP: Spontaneous bacterial peritonitis.

that there may be no benefit in early cirrhosis when there is less risk of bacterial translocation, no increase in the sympathetic nervous system activity, and when the cardiac compensatory reserve is preserved, *i.e.,* a milder splanchnic and systemic haemodynamic state[42] (Figure 2). In advancing cirrhosis however there is an up-regulation of the renin-angiotensin-aldosterone axis with salt and water preservation to attempt to compensate for a reduced effective arterial circulation (due to splanchnic vasodilatation)/cardiac output[92]. This leads to salt and water retention, and ascites formation and a loss of compensatory reserve with often hypoperfusion to organs as a result. The maintenance of effective perfusion to organs is critical especially in the face of infection, at it was proposed that at this stage the window closes and NSBBs may indeed be detrimental[21]. As cirrhosis develops however, NSBB were felt to be beneficial in reducing variceal haemorrhage and reducing bacterial translocation with an increasing sympathetic nervous system drive. The window was then felt to close as cirrhosis progresses, patients develops refractory ascites and NSBBs were thought to exert a negative impact on the cardiac compensatory reserve. With MAP being found to be an independent predictor of survival in patients with cirrhosis and ascites[93] it was proposed[21] that NSBBs by lowering MAP may indeed contribute further to hypoperfusion of organs (especially in the context of further sepsis-induced vasodilatation) leading to an increase in mortality or HRS - well known to be associated with arterial underfilling from splanchnic vasodilatation[94].

NSBB-refractory ascites cessation: The rebuttal?

The data from Sersté *et al*[20] is indeed compelling, with a change of practice recommended following years of usage of NSBBs in cirrhosis and advanced disease. One important comment is that to date there has been no RCT showing a deleterious effect of NSBBs in patients with cirrhosis and refractory ascites. There were a number of criticisms of the landmark study raised from

sections of the Hepatology community from the paper by Sersté *et al*[20]. On exploring the patient demographics in more detail, all patients who had NSBBs had confirmed varices, however in the group not receiving them only 4.1% had varices. Patients in the NSBB arm were indeed sicker with a difference in bilirubin levels (56 *vs* 48 mg/dL, $P = 0.01$), lower sodium levels and more encephalopathy which may have influenced outcome. Furthermore, the NSBB arm had more Child Pugh Grade C patients and also more patients with hepatocellular carcinoma (HCC). There was no comment in the paper about the alcohol intake of the patient cohorts, and with similar MELD and Child Pugh scores, that there may have been a potential for patients in the NSBB arm having acute alcoholic hepatitis thus accounting for the hyperbilirubinaemia, a condition with a poor prognosis. Alcohol alone has been shown to cause microvasculature obstruction and capillarization of hepatic sinusoids may lead to rises in portal pressure[95] thus introducing some potential variability into the study. Other variables between the patient cohort was that the NSBB has a lower arterial pressures ($P < 0.0001$) - which rather than secondary to the effects of NSBBs may have indeed reflected a sicker cohort of patients with lower cardiac output or indeed an impaired cardiac compensatory reserve even prior to NSBB institution. In the subset of patients (39%) who had HVPG monitoring, there was no significant difference in the HVPG in these patients 20 ($\pm$ 4.5) mmHg in the NSBB group *vs* 19.1 ($\pm$ 5) mmHg in those without NSBB. Again this leads evidence that rather than NSBB having a deleterious causative effect, the differences in patient characteristics may have had more to do with the outcomes. The next major area of interest in this paper, was that on multivariant analysis apart from use of NSBB, class of Child Pugh, HCC, aetiology of refractory ascites, renal impairment and hyponatraemia were all independent predictors of death. Interestingly however MELD score did not come out as a predictor of death despite its use in scoring patients to predict mortality[95-98] in patients with cirrhosis and on waiting lists for liver transplantation.

Recently the question of NSBB and their effects in advanced portal hypertension was studied from our own group in the United Kingdom. Leithead *et al*[22] examined the role of NSBBs on patients listed for adult liver transplantation in a single centre. In this retrospective study, 322 patients listed for liver transplantation were studied - with all patients having ascites (34.8% had refractory ascites). In a multivariate competing risk Cox model, patients on NSBB had reduced mortality compared with matched non-NSBB patients (HR = 0.55, 95%CI: 0.32-0.95, $P = 0.032$). Similarly, in the subgroup of patients with refractory ascites ($n = 117$), NSBB remained independently associated with less wait-list death (adjusted HR = 0.35; 95%CI: 0.14-0.86, $P = 0.022$). The strengths of this study included a well matched patient group, large numbers, advanced liver disease patients on the liver transplant waiting list abstinent from alcohol. It should be noted that the study groups were indeed different from that in the study from Sersté *et al*[20] with patients in this study being highly selected group, listed for liver transplantation. The study however had some criticisms including the retrospective single centre design. Selection bias must be considered in any non-randomised trial. To counter this, the study group used propensity risk score matching to try to mimimise selection bias. Patients were on lower doses of NSBB when compared to the study of Sersté *et al*[20] however another criticism was a was a lack of haemodynamic measurements in this study[22].

Leithead *et al*[22] proposed that the benefit of NSBB this may indeed be due to the reduction of bacterial translocation in patients listed for liver transplantation with ascites, and that NSBB may indeed reduce low level of the systemic inflammatory response in such patients, which in effect may reduce portal pressure too. Systemic inflammatory response (SIRS) is increasingly recognised as a pathogenetic factor in the circulatory dysfunction of advanced cirrhosis; patients with child-pugh class C disease have a greater frequency of bacterial translocation, and patients (who have ascites) with evidence of endotoxemia have more pronounced circulatory dysfunction[96,97]. Leithead *et al*[22] proposed that the window hypothesis may actually stay longer open for a subset of patients with refractory ascites. A retrospective series from another large United Kingdom transplant centre[98] reviewed patients who had refractory ascites attending for LVP. Of 114 consecutive patients, 36 patients were receiving a NSBB with no differences in survival found between the groups ($P = 0.93$). Doses of propranolol used in this study were between 40-80 mg. One important way to add information to the argument regarding the safety, efficacy and benefit of NSBBs in advanced liver disease/cirrhosis and patients with ascites would be through a meta-analytical approach. A recent review by Kimer *et al*[99] rather than perform a meta-analysis reviewed studies and their characteristics reported on the heterogeneity of the study designs and definitions of ascites. Following this, they reported on their own experience in 61 patients with cirrhosis and refractory ascites with no increase in mortality in patients on NSBBs. In the 2015 AASLD meeting, Chirapongsathorn *et al*[100] reported on a meta-analysis of 4 RCTs and 8 observational studies including 2486 patients with ascites. When compared to patients not on NSBBs, the use of NSBBs was found not to increase mortality in patients with ascites (RR = 0.94; 95%CI: 0.6-1.47, $P = 0.77$) or with refractory ascites (RR = 0.86, 95%CI: 0.47-1.57; $P = 0.63$). The use of NSBBs was not associated with death at 6 mo, 1 year or 2 years. A notable limitation is the heterogeneity of the studies included in such meta-analyses. Some patients may be late in the advanced liver cirrhosis stage (such as the cohort in our own study listed for liver transplantation[22]) and others earlier on in the window hypothesis where there indeed is a degree of cardiac compensatory reserve, and may be less likely to suffer major haemodynamic disturbances when an insult occurs such as infections (Table 3).

In a recent study by Bossen *et al*[101], data from 3 trials

Table 3 Summary of studies/recent guidelines with non-selective beta-blocker in advanced cirrhosis

Ref.	Year, country	Study design	Findings/recommendations	Strenghts/weaknesses of study (if applicable)
Bañares *et al*[57]	2002, Spain	Randomised controlled trial	More favorable reduction of HVPG comparing carvedilol with propranolol however an increase in diuretic requirement in patients on carvedilol suggesting potential worsening of ascites	Increased requirement of diuretic not a hard end-point
Sersté *et al*[20]	2010, France	Single centre observational prospective case study	Patients on NSBB in refractory ascites having higher 1-year mortality than those not on NSBB	Non-randomised Lack of haemodynamic data. No competing risk analysis
Mandorfer *et al*[90]	2014, Australia	Single centre retrospective study	NSBB associated with higher transplant free survival but increase in renal dysfunction and mortality following episode of SBP	Groups not well matched at baseline with NSBB group having higher bilirubin in subgroup analysis
Leithead *et al*[22]	2015, United Kingdom	Single centre, retrospective case study	NSBB associated with reduced wait-list mortality and a higher likelihood of survival to transplantation	Lack of haemodynamic measurements. Non randomized. Well matched groups
Tripathi *et al*[67]	2015, United Kingdom	British guidelines	NSBB to be continued till episode of SBP, hypotension of renal failure (based on level 2b, Grade B evidence)	National guidelines based on all available evidence
Kimer *et al*[99]	2015, Denmark	61 patients with cirrhosis and ascites (following a review of 14 trials)	No survival difference in patients on/not NSBBs in patient cohorts with ascites	Small retrospective analysis
de Franchis[25]	2015, International	Meeting consensus statements	NSBB dose reduction or discontinuation can be considered if hypotension/ hyponatraemia or renal function impairment in patients with refractory ascites. If a clear precipitant for these (*e.g.*, SBP), NSBB can be restarted once parameters normalised	International consensus statements based on evidence
Robins *et al*[98]	2014, United Kingdom	Letter - retrospective review of 114 patients undergoing LVP	No significant difference in survival comparing patients on NSBB and those not	Small retrospective series
Bossen *et al*[101]	2015, Denmark and France	Post-hoc analysis of 3 RCTs	NSBBs not associated with increase in mortality in patients with cirrhosis and ascites Cessation of NSBB linked thereafter to increase in mortality due to liver decompensation events	Multicentre trials, 3 RCTs, large data set and reflective of real world experience. Lack of haemodyamic studies and assessment of severity of portal hypertension. NSBBs stopped during admission so? true reflection of their effects on mortality

HVPG: Hepatic venous pressure gradient; SBP: Spontaneous bacterial peritonitis; LVP: Large volume paracentesis; NSBB: Non-selective beta-blocker; RCTs: Randomised controlled trials.

of 1198 patients was reviewed in a post-hoc analysis to assess the effect of the use of NSBBs on mortality in patients with cirrhosis including the subgroup who had ascites. A cox-regression analysis was performed to assess for mortality once correcting for variables between groups of patients on NSBBs (n = 559) compared to those not (n = 629). The data were taken from 3 trials conducted to assess the safety of satavaptan in treating ascites. Two hundred and forty patients had diuretic refractory ascites requiring regular paracentesis - although the definition and categorization of refractory or diuretic responsive ascites was down to independent clinicians per site. The important finding from such a well-constructed, rigorous and clinically relevant study/ analysis was that NSBBs did not increase mortality or hospitilisation in patients with cirrhosis or in the subgroup of those with refractory ascites. Although there were no portal pressure measurements performed and a lack of data on the presence of varices (thus lack of markers of severity of portal hypertension), this was a real world practice/experience. The authors also tried to address the sub-groups who have differences in systemic haemodynamics within the window hypothesis based on MAP with no obvious effect on mortality between groups. Interestingly during the follow-up period, 29% of those on NSBB at the beginning of entry to trials stopped these, reflecting possible current day practice. This cessation of NSBB was thereafter linked to a sharp increase in mortality and coincided with not only hospitalization but also variceal bleeding, bacterial infection, and/or development of the HRS. Despite being a well conducted trial with actual clinical data, it should be noted that only 133 patients had a MELD score > 18, thus may not actually have been further down the window hypothesis pathway. Furthermore, as the NSBBs were stopped mid-way through study/admission it is difficult to conclude mortality would/would not have differed if they had actually been continued to the end-points stated. Patients on NSBBs had a median MAP of 83 mmHg which was similar to the group who were

not on a NSBB and therefore are not representative of the group previously shown to be harmed by the use of NSBBs. The authors concluded that discontinuation of NSBBs increased the mortality by over 5 times. It is more biologically plausible that this increase is due to the reasons NSBBs were discontinued in the first place, *i.e.,* hospitalization, infections and bleeding. It is likely that NSBBs would have impaired the cardiovascular reserve during these episodes thus contributing to the higher mortality. This relationship could have been demonstrated by close monitoring of MAP of patients on NSBBs after their discontinuation. This is a major limitation of studies performing post hoc analysis.

RECENT ADVANCES AND FUTURE DIRECTIONS OF NSBBs IN LIVER DISEASE

NSBBs: Bacterial translocation and effect in infections

The role of NSBBs in advanced cirrhosis certainly needs further evaluation. A large multicentre trial looking at the beneficial or deleterious effects of NSBBs in outcome of patients with advanced portal hypertension, especially in patients with ascites is clearly required. With the data suggesting that NSBBs have a deleterious effect after 1st episode of SBP[90], but with conflicting animal data suggesting NSBBs may indeed reduce bacterial translocation across the gut[59] the optimal timing of administration of NSBBs and their role in infection prevention or clinical deterioration needs to be clarified. Another issue is whether in patients who are at high risk of SBP as per the criteria outlined by Fernández *et al*[102] (child-pugh score ≥ 9 points with serum bilirubin level ≥ 3 mg/dL) or impaired renal function (serum creatinine level ≥ 1.2 mg/dL, blood urea nitrogen level ≥ 25 mg/dL, or serum sodium level ≤ 130 mEq/L) in whom primary prophylaxis is recommended should have their NSBBs discontinued or not, and also the optimal timing thereafter for reintroduction? Current BSG guidelines[67] suggest that NSBB are indeed discontinued at time of first advent of SBP however the lead up to this in those at high risk is not clear, and the potential benefits of NSBB in prophylaxis of variceal haemorrhage and reduction of potential low-grade SIRS/bacterial translocation need to be weighed up against the development of SBP. Clinical judgment however is required with reintroduction of NSBB once an acute septic hit has resolved. In patients with refractory ascites, the Baveno Ⅵ guidelines[25] state that NSBBs should be discontinued if hypotension (systolic blood pressure < 90 mmHg, hyponatraemia (< 130 mEq/L) or AKI. They state if a clear precipitant such as SBP (or gastrointestinal bleed) than NSBB should be considered to be restarted once the parameters cited normalise - however the grading of evidence for these statements was 5:D (expert opinions based on non-systematic reviews) - thus it should be interpreted with caution. Also cessation of NSBB in infective states such as SBP should be done with caution as SBP can increase portal pressure itself *via* bacterial translocation and thus a pro-inflammatory release at a sinusoidal level with consequent rise in portal pressure[103]. Rather than cessation of NSBB completely, in the author's opinion continuation with close observation may be a preferable strategy, although further trials are warranted for this approach. Potentially stratifying patients into risk of SBP based on not only the Fernández *et al*[102] criteria, but using variceal assessment and HVPG (or non-invasive portal pressure studies) may allow a future study to look into randomising patients to NSBB alone, NSBB with primary prophylaxis of antibiotics or antibiotics alone, and then following patients up for the advents of SBP and variceal haemorrhage.

NSBBs and portal vein thrombosis

Another area to be studied is the effect of NSBBs on portal blood flow, and whether this could lead to portal vein thrombosis (PVT) due to stagnant blood flow within the portal vein. Qi *et al*[104] discussed the hypothesis of NSBB potentially causing a reduction of portal vein inflow however with a lack of any large trials this area is still hypothetical. To date, one small unpublished study[105] found in 56 cirrhotic patients (with no HCC) who had an ultrasound every 6 mo on follow-up, on multivariant analysis the use on NSBB was an independent predictor of PVT (OR = 3.3, 95%CI: 1.4-6.8, $P < 0.001$). In another study published in abstract form retrospectively studied a large cohort of 568 patients assessing for factors predictive of the development of PVTs[106]. Although only 23 patients developed PVT, on mutlivariant analysis NSBBs were a risk factor for development of PVT (OR = 4.3, 95%CI: 1.4-12.6; $P = 0.01$). Larger published studies however are needed to explore this association in more detail, with ideally HVPG measurements.

NSBBs and HVPG

One area pertaining to NSBB research is indeed the measurement of HVPG and guided response when using any medical therapies - especially NSBBs. This modality is only reserved in specialist centres, often in a research setting only. The absence of assessment of haemodynamic response remains a criticism levelled at multiple research papers in the field, even those that have suggested changes in clinical practice in patients on NSBBS[20,22]. The role of NSBB - especially carvedilol with its more potent effect than propranolol in primary prophylaxis in small varices or even the prevention of variceal formation is not clear as yet, and longitudinal studies are required in this field, to see if the NSBB window for opening can be extended earlier in the disease course. Also studies comparing carvedilol with the other NSBBs are required in both a primary and secondary prophylaxis setting and in patients with advanced cirrhosis. If patients are diagnosed with non-invasive evidence of portal hypertension from imaging, blood work or elastography methods (after endoscopic verification of no varices or small varices)[107] a RCT is needed to assess intermittently the development of

varices comparing propranolol, carvedilol, nadolol and placebo. A recent systematic review and meta-analysis of 5 studies comparing carvedilol and propranolol suggested better haemodynamic reduction profile of carvedilol however commented on the lack of "quality" of the trials[108].

Other areas of interest include the role of combined different types of NSBB with VBL after a variceal haemorrhage compared to NSBB alone, to attempt to show what is the optimal therapy for prevention of rebleeding. In a trial from Egypt[109] published in abstract form, propranolol was studied in the prevention of recurrence of varices after endoscopic eradication. Ninety patients who had varices eradicated (primary and secondary prophylaxis) were divided into just follow-up alone (n = 43) or propranolol (n = 47). Propranolol use was associated with a delay in time to recurrence of varices, but not in the recurrence of varices. Also teasing out which NSBB reduces rebleeding rates superiorly is indeed required in a potential trial. A combined or an additive approach needs to be studied further, where if there is a failure to reduce size of varices (or reduce HVPG) by either banding or NSBBs alone, and assessing whether an addition of the other modalities improves HVPG reduction and rebleeding rates. To explore these issues in more detail, well-constructed likely multicentre RCTs large studies are required. This not only applies to oesophageal varices but gastric varices as well when patients cannot be entered necessarily into a band ligation programme after a herald bleed, thus more interest in NSBB could be applicable in prevention of gastric variceal rebleeding - and comparison of different NSBBs.

NSBBs and alcoholic hepatitis and ACLF

Another potential area of interest could be the role of NSBBs in patients with acute alcoholic hepatitis, one of the most florid manifestations of liver disease. Although in the studies from Plevris *et al*[70] there was no benefit in prevention of 1st variceal bleed in a cohort of patients with chronic liver disease, assessment of levels of pro-inflammatory cytokines, and even gut bacterial translocation rates in experimental models of alcoholic hepatitis when NSBBs administered and when not would be interesting. As the mice models have shown, NSBBs can significantly lower portal pressures, but also speed up intestinal transit times and also lower rates of bacterial overgrowth and translocation[59]. In this study propranolol was used, thus the role of carvedilol would be interesting. This could then be translated to an alcoholic hepatitis patient cohort with measurements of portal pressure and pro-inflammatory cytokine release, and to assess if NSBBs had a role in prevention of HRS or worsening of it. A recent retrospective study from Sersté *et al*[23] tried to answer this question, identifying 139 biopsy proven patients with alcoholic hepatitis with 34.5% receiving a NSBB. These patients had lower heart rates, MAP but comparable MELD cores and Maddrey discriminant functions to the non-NSBB arm. There was a higher 168-d cumulative incidence of AKI found in the NSBB group (P = 0.0001) however similar 168-d transplant free survival between the groups. Thus it may well be that patients with Alcoholic Hepatitis and a marked SIRS component with marked systemic vasodilatation may not indeed benefit from NSBBs, whereas another subgroup where bacterial translocation (in the earlier stages of disease) can be reduced may benefit having NSBBs continued. The effect of NSBBs on SIRS was studied in a high profile study from Mookerjee *et al*[24], this time in patients with ACLF. In this prospective observational study, 349 patients were studied with 47% receiving NSBBs. The advent of ACLF was observed with lower rates of ACLF in patients at presentation (P = 0.047) who were on NSBBs. On follow-up patients on NSBBs had a better 28-d survival [estimated risk reduction 0.596 (95%CI: 0.361-0.985; P = 0.0436)] and improvement in survival was associated with a significantly lower white cell count [NSBB: 8.5 (5.8); no NSBB: 10.8 (6.6); P = 0.002] suggesting those on NSBBs may either be more effective in those patients who have lower levels/grade of SIRS or may potentially reduce SIRS *via* effects on bacterial translocation in ACLF patients. A major limitation of this study is the lack of methods to control for differences in baseline characteristics between NSBBs and non-NSBBs groups, such as propensity score matching. A significant proportion of patients discontinued NSBBs for reasons that are not clear.

NSBBs and HCC

NSBBs, by way of potential reducing bacterial translocation and also reducing levels of SIRS may indeed have a hypothetical benefit in reducing the portal load of danger signals/molecules from the gut to the liver, with a potential benefit in altering the cascade in development of HCC. HCC is known to be linked to bacterial translocation and liver inflammation through Toll-like receptor signalling[110], thus one could propose NSBBs may have a beneficial effect in preventing signalling and translocation. There is a clear association between the inflammatory cascade and HCC formation[111] thus reducing the bacterial translocation stimulus for inflammation could be an important step in cancer prevention. At experimental level, NSBBs have also been shown to inhibit key processes involved in tumour development such as decreasing angiogenesis by inhibiting vascular endothelial growth factors, and by blocking adrenergic-mediated stimulation that can promote angiogenesis[112]. Beta-blockers too may block cell proliferation, migration, invasion, resistance to programmed cell death and metastasis too[113,114] and have been shown to improve the effect of some chemotherapeutic agents[115]. To this end a systematic review from Thiele *et al*[116] recently examined 23 trials on 2618 patients with cirrhosis to see if there was a link between patients on NSBB and reduction in incidence of HCC. The study found that NSBBs did not reduce HCC related mortality. Of the 47 of 694 (NSBB arm) developed HCC *vs* 65 of 697 controls (risk difference -0.026; 95%CI: -0.052 to -0.001; number needed to treat 38 patients). This area certainly requires further research.

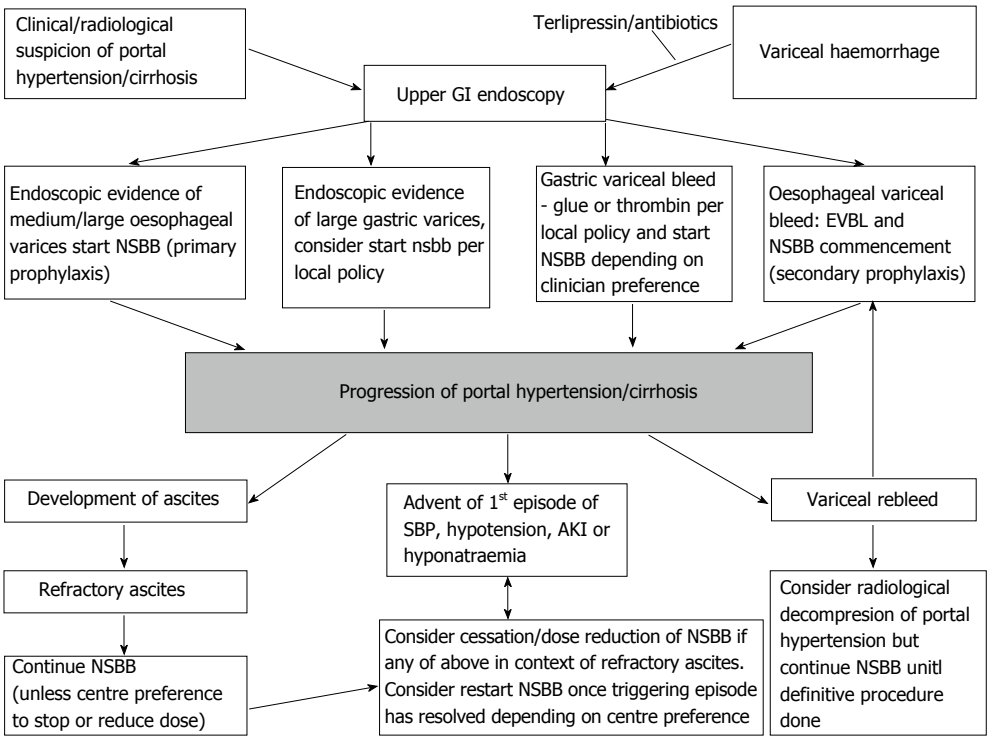

Figure 3 Proposed algorithm of Non-selective beta-blocker usage based on current guidelines and recent papers. NSBB: Non-selective beta-blocker; GI: Gastrointestinal; SBP: Spontaneous bacterial peritonitis.

NSBBs and stratification of patients per window hypothesis

Further complex mathematical modelling may indeed be required to retrospectively delineate cohorts of patients however this will be very difficult given the trials/study designs and captured data already gained. A well designed RCT however with long term follow-up assessing for markers of SIRS, portal pressure assessments, physiological parameter assessments, recording of septic insults, and cardiac function studies (ideally non-invasively) may add more information in the future. In a recent abstract the role of myocardial dysfunction was investigated by Giannelli *et al*[117]. In 583 patients undergoing liver transplantation assessment, 34% had refractory ascites. Patients had invasive cardiac assessment in including a right heart catheterization (right and left ventricular stroke work index assessments). Patients with refractory ascites had a significantly lower MAP, heart rate and a higher HVPG than those without refractory ascites as well as lower right and left heart stroke work index assessments. NSBBs were associated with a significant drop in the left ventricular stroke work index in patients with refractory ascites compared to those not on NSBBs, however there was no difference noted between the 2 groups in patients without refractory ascites. These findings may support the original window hypothesis[21] that NSBBs may indeed have a negative effect on the cardiac compensatory reserve in advanced cirrhosis (listed for transplantation in the study from Giannelli *et al*[117]. For future studies non-invasive modalities measuring cardiac function (*e.g.,* functional Magnetic resonance imaging) in cirrhosis may be more helpful to tease out the cardiovascular shifts occurring as liver disease progresses in patients with ascites and on NSBBs.

The effect of NSBBs in patients with cirrhosis affecting acute insults such as the development of HRS or AKI is an important area following on from the Mandorfer *et al*[90] study. Future studies describing or assessing outcomes of patients on NSBBs within different phases of the window hypothesis clearly need to examine these acute insults that lead to hospital admission of patients. In a recent multicenter study[118] from the North American Consortium for the Study of End Stage Liver Disease, 981 patients with cirrhosis admitted to hospital were studied. It was found that patients on NSBBs developed AKI compared to those not on NSBBs (49% *vs* 41%, $P = 0.019$), however whilst NSBBs were indeed found by backward elimination regression analysis to be associated with development of AKI during admission, there was no association with death during admission. The advent of infection in those admitted and on a NSBB was associated with AKI compared to those without infection ($P < 0.05$). Thus as per recommendation within Baveno Ⅵ, there may indeed be value to temporarily stop NSBBs in patients who are admitted with a complication of cirrhosis to avoid the issue of hypoperfusion of the kidneys in the face of a potentially impaired compensatory cardiac reserve, thus avoiding the advent of AKI and development of HRS (Figure 3).

CONCLUSION

In conclusion, it is an exciting time for NSBBs in patients

with liver disease. Their role has been firmly established over the years in prevention of variceal haemorrhage and rebleeding. It has however become clear that in certain stages of liver disease their benefit may become outweighed by their deleterious effects on systemic haemodynamics. It is clear that in a subset of patients, continuing NSBBs may indeed be appropriate to prevent variceal haemorrhage, SBP and improve outcomes, however when patients begin to deteriorate with sepsis in later disease or other evidence of end-organ hypoperfusion, then that may indeed be the time that the NSBB window closes. More studies are indeed required to tease out this exact timing for cessation, and also to expand the potential beneficial roles for NSBBs in the future. Not only does the window for NSBBs' beneficial effects open, but with the recent conflicting data as their role in advanced cirrhosis, the debate as to when to stop NSBBs has indeed opened too. There is an urgent need for well designed prospective studies of NSBBs in patients with advanced liver and in the setting of SBP to define clinical parameters for the safe administration of these indispensable treatments for portal hypertension.

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REVIEW

Allosteric modulation of cholinergic system: Potential approach to treating cognitive deficits of schizophrenia

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Abstract

Schizophrenia is a psychiatric disorder affecting approximately 1% of the population worldwide and is characterised by the presence of positive and negative symptoms and cognitive deficits. Whilst current therapeutics ameliorate positive symptoms, they are largely ineffective in improving negative symptoms and cognitive deficits. The cholinergic neurotransmitter system heavily influences cognitive function and there is evidence that implicates disruption of the central cholinergic system in schizophrenia. Historically, targeting the cholinergic system has been impeded by poor selectivity leading to intolerable side effects warranting the need to develop more targeted therapeutic compounds. In this review we will summarise evidence supporting the roles of the cholinergic system, particularly the muscarinic M_1 receptor, in the pathophysiology of schizophrenia and discuss the potential of a promising new class of candidate compounds, allosteric ligands, for addressing the difficulties involved in targeting this system. The body of evidence presented here highlights the dysfunction of the cholinergic system in schizophrenia and that targeting this system by taking advantage of allosteric ligands is having clinically meaningful effect on cognitive deficits.

Key words: Central nervous system; Antipsychotic; Allosteric; Cholinergic; Schizophrenia; Mutagenesis; Cognition; Muscarinic

Core tip: Schizophrenia is a psychiatric disorder affecting approximately 1% of the world population. Current treatments inadequately redress the cognitive impairments associated with the disease. In light of that we discuss the role of the cholinergic system, in particular the muscarinic M_1 receptor, in schizophrenia and cognition and how allosteric compounds are being developed to address this undertreated aspect of the disease. We also discuss and compile mutagenesis studies of the muscarinic M_1 receptor and how they relate to allosteric binding and function.

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INTRODUCTION

Cognitive impairment is associated with multiple disease states and usually has a big impact on quality of life. Schizophrenia is a psychiatric disorder with a predominant age of onset from late teens to early adulthood that affects approximately 1% of the world population. It is diagnosed by the presence of what are characterised as positive and negative symptoms and cognitive deficits. Unlike neurological disorders no major lesion was apparent in the central nervous system (CNS) from subjects with schizophrenia[1] and therefore many of the hypotheses as to its cause have been based on the actions of drugs that either worsen or ameliorate the symptoms of the disorder.

The dopamine hypothesis

With the serendipitous discovery that chlorpromazine had unexpected therapeutic benefit when given to psychotic individuals there was a significant effort to develop a range of what became known as first generation neuroleptic drugs[2]. Subsequently, it was determined that the ability of these drugs to reduce the severity of positive symptoms in some people with schizophrenia was directly related to the ability of first generation neuroleptic drugs to act as antagonists at the dopamine D2 receptor[3]. Understanding this mechanism led to the dopamine hypothesis of schizophrenia which states hyperactivity of the dopamine neurotransmitter system contributes to the pathophysiology of schizophrenia (reviewed in[4]).

The dopamine hypothesis was the first attempt to encapsulate the biological mechanisms underpinning schizophrenia. This hypothesis grew from an understanding that the early antipsychotics block the dopamine D2 receptor and that drugs which increase CNS dopamine function cause or worsen psychosis. The original dopamine hypothesis proposed that widespread hyperactivity of the dopamine system within the brain was the cause of psychotic symptoms but the hypothesis was subsequently refined to propose that cortical hypo-activity in addition to subcortical hyper-activity of dopamine contributed to both the psychotic and other symptoms associated with schizophrenia (reviewed in[5]). Inconsistent reports of differences in dopamine receptor levels, metabolic enzymes[6], and imaging studies have led to questioning of the dopamine hypothesis[7,8] and investigation into other possible mechanisms of disease aetiology. However, a neuroimaging report of increased amphetamine-induced dopamine release in the striatum of subjects with schizophrenia appears to reinforce a role for dopamine in the aetiology of the disorder (reviewed in[9]).

The serotonin hypothesis

Clozapine is the archetypal drug that led to the development of a family of drugs termed second-generation neuroleptics[10]. Whilst the second-generation drugs were developed to bind to some of the receptors targeted by clozapine it is notable that none of these drugs have achieved the extended therapeutic reach of clozapine such as improving the cognitive deficits[11]. An early second generation neuroleptic drug, risperidone, was suggested to have significant benefits compared to first generation neuroleptic drugs[12] which were presumed to be due, at least in part, to its ability to antagonise the serotonin 2A receptor[13]. The clinical benefit obtained by risperidone, added to the observation that drugs such as lysergic acid diethylamide could cause psychotic symptoms by stimulating the serotonergic system[14], became the evidence to support the serotonin hypothesis of schizophrenia.

Initial interest in the serotonergic system stems from the observation that drugs affecting serotonin receptors produce psychotomimetic effects[15] and the discovery that second generation neuroleptics bind to various serotonin receptors[16,17]. These observations have led to investigation of the serotonin system both post-mortem and *in vivo*; a recent meta-analysis[18] demonstrated a moderately higher level of the serotonin receptor subtype 5-HT_{1A} and a substantially lower levels of the 5-HT_{2A} receptor subtype in the prefrontal cortex of post-mortem subjects with schizophrenia.

The glutamate hypothesis

The glutamate hypothesis is somewhat unique as it is founded in the observation that phencyclidine could cause a broad range of symptoms in normal individuals[19] and exacerbate psychotic symptoms in patients with schizophrenia[20] but no drug targeting the glutamatergic system has proved useful in treating the disorder[21]. Further investigation has revealed elevated glutamate activity particularly in the medial prefrontal cortex and basal ganglia of un-medicated and first episode patients, demonstrated by magnetic resonance spectroscopy studies (reviewed in[22]). Of studies in post-mortem brain tissue, the most robust finding has been widespread differences in glutamatergic pyramidal neuron morphology, particularly lower numbers[23] and lengths of dendrites[24] and lower numbers of dendritic spines[25] in deep cortical layer Ⅲ of the dorsolateral prefrontal cortex. There have also been mixed reports of differences in mRNA and protein levels of receptor subunits, transporters, and synthesis enzymes (post-mortem glutamatergic differences in schizophrenia reviewed in[26]).

The cholinergic hypothesis

Preceding all of these neurotransmitter hypotheses was the cholinergic hypothesis which was founded on the observation that some subjects with schizophrenia had remission or an improvement in symptom severity

after coma induced by a dose of acetylcholine (ACh)[27]. Since those early experiments components of the cholinergic system, the muscarinic receptors, have been targeted by drugs such as clozapine and olanzapine that reduce the symptoms of schizophrenia[28] and drugs such as benzatropine which were used to control the extrapyramidal side effects associated with treating with first generation antipsychotic drugs[29]. Whilst these neuropsychopharmacological findings are important to consideration of the role of the cholinergic system in schizophrenia, other studies which have advanced our understanding of the cholinergic system and how it may be affected in the disorder have been important in refining the cholinergic hypotheses of schizophrenia.

The cholinergic system consists of several distinct pathways; the most well studied being the pathway that projects from the basal forebrain to most of the CNS[30]. This pathway extensively modulates the dopamine system by affecting striatal dopamine release[31]; the glutamate system by potentiating NMDA receptors[32]; and the serotonergic system *via* projections to the dorsal raphe nucleus where ACh inhibits the release of serotonin[33] (cholinergic interactions in schizophrenia reviewed in[34]). Whilst the modulatory interactions of the cholinergic system with other neurotransmitter systems provide mechanisms which can implicate that system in schizophrenia there are also several lines of evidence to suggest that the cortical muscarinic M_1 receptor, a component of the cholinergic system, is particularly implicated in the pathophysiology of schizophrenia.

Although there have been several reviews discussing the cholinergic system and its role in schizophrenia[35,36], there have recently been some exciting advances in developing drugs to target the cholinergic system[37-39] that make a review of the area timely. This is particularly the case because the cognitive deficits of schizophrenia are largely non-responsive to current drug treatments[40] whereas preliminary data suggest that the new drugs targeting the cholinergic system will improve cognition[41]. Cognitive deficits are a core feature of schizophrenia and are made up of deficits in the domains of speed of processing, attention/vigilance, working memory, verbal learning and memory, visual learning and memory, reasoning and problem solving, and social cognition (reviewed in[42]). The impact of these deficits in people with schizophrenia is now considered the most debilitating of all the symptom domains associated with the disorder. Therefore, this review will first summarize the cholinergic system, then briefly review evidence supporting its role, particularly the muscarinic M_1 receptor, in cognition and the pathophysiology of schizophrenia and finally discuss the potential of a promising new class of candidate compounds and how they target the muscarinic M_1 receptor for addressing the difficulties involved in targeting this system.

OVERVIEW OF THE CHOLINERGIC SYSTEM

ACh is a modulatory neurotransmitter[43] in the CNS and peripheral autonomic and somatic nervous systems. It is essential to the function of all branches of the peripheral nervous system[44] and has been associated with a large number of cognitive processes in the CNS. Two classes of integral membrane receptor mediate signal transmission: The ionotropic nicotinic receptors (nAChR), and the metabotropic muscarinic receptors.

Within the CNS, cholinergic neurons are predominantly located in the basal forebrain as cortical projection neurons (reviewed in[45]) and striatal interneurons (Figure 1). The projection neurons arise from four cell groups (Ch1-4) in the basal forebrain[46] and from the pedunculopontine-lateral dorsal tegmental area in the brainstem. These cell groups are not exclusively composed of cholinergic neurons, but contain a diverse mix of other neurotransmitter systems including GABAergic, peptide transmitter, and catecholaminergic neurons. These projection neurons modulate all regions of cortex including the hippocampal formation[46]. In addition to the projection nuclei of the basal forebrain, striatal cholinergic interneurons interact heavily with the dopaminergic projections from the substantia nigra pars compacta and the ventral tegmental area (VTA)[47] (The human cholinergic brain architecture is extensively reviewed in[30]).

ACh acts in part *via* the family of five muscarinic (M_{1-5}) receptors; they have a differential expression pattern throughout the human CNS[48]. Point mutation and other studies suggest that the structural position of the muscarinic receptor transmembrane domains relative to one another affect the differential binding specificity of ligands to these receptors[49,50]. Significantly, the orthosteric site on the five muscarinic receptors is highly conserved and this has presented a challenge to the development of receptor-specific drugs.

Muscarinic M_1 receptors

Of the five muscarinic receptors, the muscarinic M_1 receptor is the predominant muscarinic receptor in all cortical areas[48] where it is located on excitatory neurons to modulate their firing, for example, by potentiating NMDA receptors[32] and at cholinergic synapses[51]. Mouse knockout and knock down studies have elucidated some of its roles in CNS function. For example, in muscarinic M_1 receptor knockout mice, mitogen activated protein kinase (MAPK) signalling is impaired in cortical neuronal cultures[52] and hippocampal slices[53]. The role of MAPK in hippocampal long term potentiation[54] and neuronal plasticity[55] demonstrate a potential mechanism by which muscarinic M_1 receptors modulate learning and memory. Gamma oscillations (20-80 Hz) of neuronal firing patterns are associated with memory[56], hippocampal gamma oscillations induced by muscarine, a muscarinic agonist, but not those induced by kainite, a glutamatergic kainite receptor agonist, are completely absent from muscarinic M_1 receptor knockout mice[57]. Another study reported that muscarinic M_1 receptor knockout mice had fewer and shorter dendrites and disrupted cortical tonotopic maps in the auditory cortex[58]. Additionally,

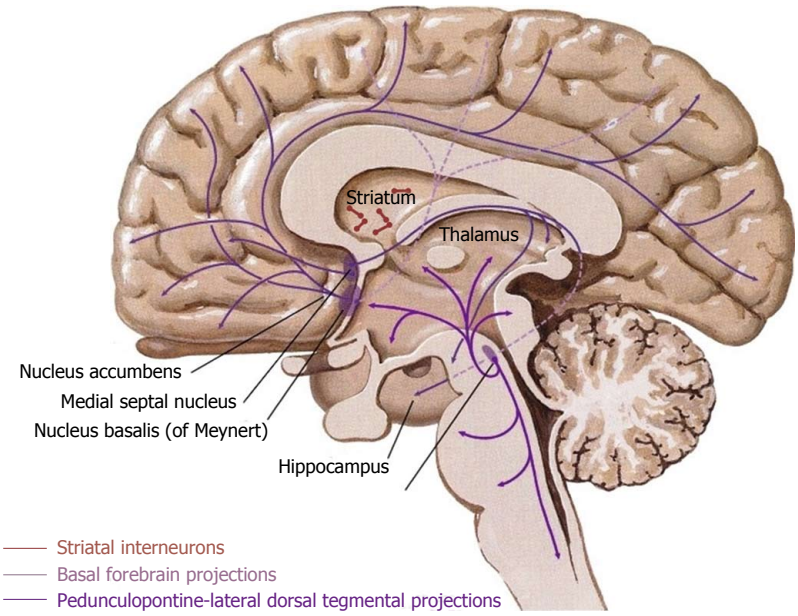

Figure 1 Schematic diagram of the human cholinergic pathways. Adapted from[112].

muscarinic M_1 receptor knockout mice had impaired experience dependent plasticity in the auditory cortex[59], implicating the muscarinic M_1 receptor in auditory cortical organisation, sensory processing, and learning. Additionally, muscarinic M_1 receptor knockout mice are deficient in working memory and memory consolidation processes where the hippocampus and cerebral cortex interact[60]. These studies together implicate muscarinic M_1 receptors in various aspects of learning and memory.

In addition to their cognitive effects, muscarinic M_1 receptors are implicated in the response to a number of brain-penetrant drugs in mice. Muscarinic M_1 receptor knockout mice are highly resistant to seizures induced by pilocarpine, a muscarinic agonist, which does not supress the voltage dependent K^+ M current in these animals. Conversely, muscarinic M_{2-4} receptor knockout mice display wild-type seizure response and M current suppression when administered pilocarpine[61]. As M current suppression is considered the mechanism by which pilocarpine seizures are produced[62], the muscarinic M_1 receptors mediate this drug induced seizure response by modulating M currents in sympathetic neurons[61]. Muscarinic M_1 receptor knockout mice displayed an elevated striatal level of extracellular dopamine and increased locomotor activity, which neuroleptic treatment attenuated[63]. In addition, the mice had an increased sensitivity to amphetamine administration, in both the locomotor response and striatal dopamine levels[63] demonstrating an interaction between the cholinergic and dopaminergic systems. This interaction is supported by the report of an increase in morphine-induced analgesia and lower rates of self-administration of morphine and cocaine in muscarinic M_1 receptor knockout mice[64]. Taken together, these studies implicate the muscarinic M_1 receptor in the both the response to these drugs and as a mechanism by which the cholinergic system interacts with the dopamine reward pathway.

THE CHOLINERGIC SYSTEM IN THE PATHOPHYSIOLOGY OF SCHIZOPHRENIA

The cholinergic system's role in schizophrenia is supported by research involving neuropsychopharmacological agents, post-mortem brain tissue, and neuroleptic drug treatments. In addition to the these three areas of research, a landmark single photon emission computed tomography (SPECT) study showed widespread lower levels of muscarinic receptor binding in the cerebral cortex in patients with schizophrenia who were medication free compared to healthy control subjects[65].

Neuropsychopharmacology

Several neuropsychopharmacological studies provide evidence to support the role of muscarinic receptors in schizophrenia. Administering a muscarinic antagonist, scopolamine, to healthy control subjects produced deficits in spatial and working memory as well as sustained visual attention[66]. In another report this group demonstrated that scopolamine produced deficits in spatial and object working memory using the n-back paradigm[67], which is used to assess working memory function in patients with schizophrenia. Additionally, acute administration of scopolamine to healthy subjects produced deficits in maintenance of working memory[68]. Another study demonstrated higher doses of scopolamine as well as atropine and Ditran, two other muscarinic antagonists, produced hallucination, and delirium in addition to cognitive deficits in healthy

Table 1 Muscarinic receptors in post-mortem studies

Ref.	Receptor Type		Regions	Results
	Protein	mRNA		
[114]	M_1/M_4		CPu	Lower binding
[115]	M_2/M_4		CPu	Lower binding
[116]		M_1 M_2	CPu	M_1 no difference; M_2 below sensitivity in both SCZ and HC
[117]	M_1/M_4		PFC	Lower binding
[76]	M_1/M_4	M_1 M_4	DLPFC; PCx	Lower binding and M_1 mRNA levels in DLPFC; no difference in binding, lower M_1 and M_4 mRNA levels in PCx
[118]		M_1	PMC	Lower levels
[119]	M_1/M_4		ACC	Lower binding
[120]	M_2/M_4		ACC	No difference
[121]	M_1/M_4 M_2/M_4		STG	Lower M_1/M_4 binding non-significant; lower M_2/M_4 binding
[77]	M_2 M_3	M_2 M_3	DLPFC; PCx	M_2 and M_3 binding and M_3 mRNA no difference; M_2 mRNA below sensitivity in both SCZ and HC
[122]	M_1/M_4	M_1 M_4	Hipp	Lower binding; lower M_4 mRNA no difference in M_1 mRNA levels
[123]	M_1/M_4 M_3		PMC	Lower M_1/M_4 binding; no difference in M_3 binding
[78]	M_1		DLPFC	Lower binding in subset of SCZ subjects
[81]	M_1 M_3	M_1 M_3 M_4	PMC	Lower M_1 binding in subset of SCZ subjects; no difference in mRNA levels or M_3 binding
[82]	M_1		DLPFC; ACC; BrA	Lower binding in subset of SCZ subjects

"/" Indicates that the radioligand used in the study binds both receptor types listed. CPu: Caudate-putamen; SCZ: Subjects with schizophrenia; HC: Non-psychiatric healthy control; PFC: Prefrontal cortex; DLPFC: Dorsolateral prefrontal cortex; PCx: Parietal cortex; PMC: Premotor cortex; ACC: Anterior cingulate cortex; STG: Superior temporal gyrus; Hipp: Hippocampus; BrA: Broca's area.

control subjects[69]. Thus blocking muscarinic recptor activity mimics both positive and cognitive symptoms observed in patients with schizophrenia.

Sleep disturbances, including impaired sleep continuity, abnormal REM latency[70] and decreased slow wave sleep[71] observed in patients with schizophrenia have been associated with impaired sleep dependent memory consolidation, as well as negative symptom severity[71]. Administration of cholinergic agonists to healthy volunteers causes shortening of REM latency during sleep[72,73] and altered REM latency in a subset of patients with schizophrenia more severely than healthy control subjects[74]. These findings demonstrate that pharmacological manipulation of the cholinergic system can mimic and worsen the sleep disturbances observed in patients with schizophrenia. These studies add support to the body of evidence implicating the cholinergic system in the pathology of schizophrenia.

Post mortem studies

A role for the muscarinic M_1 receptor in schizophrenia is supported by a variety of post-mortem studies investigating radioligand binding and mRNA levels in multiple brain regions (Table 1), which report a decrease in muscarinic M_1/M_4 receptor binding and muscarinic M_1 receptor mRNA levels in multiple brain regions (reviewed in[75]). Notably, we reported a decrease in muscarinic M_1[76], but not M_2, M_3[77] or M_4[76] receptor protein in the cortex of subjects who had schizophrenia confirming that the M_1 receptor is selectively decreased in people with the disorder. More recently, we[78] reported lower binding of [^{3}H]pirenzepine ([^{3}H]PRZ), a muscarinic M_1/M_4 receptor selective antagonist, in the dorsolateral prefrontal cortices from people with schizophrenia to be limited to a subset (approximately 25%) of subjects; these subjects had 75% lower binding to muscarinic receptors in Brodmann area (BA) 9 when compared to both healthy control subjects and other subjects with schizophrenia. These data underpin the proposal of a subgroup within schizophrenia that have a muscarinic receptor deficit schizophrenia (MRDS). This hypothesis is pertinent to the growing acceptance that schizophrenia is a syndrome of disorders that may well have differing aetiologies[79].

Although [^{3}H]PRZ binds to both muscarinic M_1 and M_4 receptors, its selectivity for the muscarinic M_1 receptor was increased under the conditions used for the study that identified MRDS[80]. Furthermore, we previously showed that muscarinic M_1, but not M_4, receptor mRNA and [^{3}H]PRZ binding levels were significantly lower in BA 9 in subjects with schizophrenia[76]. These data, combined with the high abundance of muscarinic M_1 receptors relative to the other muscarinic receptor types within the cerebral cortex[48], strongly implicates muscarinic M_1 receptors in the pathophysiology of the MRDS group. More recent work has demonstrated lower [^{3}H]PRZ binding in BAs 6, 10, 24, 44 and 46 in the same cohort[81,82]; these findings are consistent with the widespread loss of cortical muscarinic receptors in people with schizophrenia reported in the SPECT study[83].

Neuroleptic drug treatment

Traditional antipsychotic therapies achieve their action by blockade of central dopamine D2 receptors (reviewed in[4]); unfortunately, these drugs can cause extrapyramidal side effects (EPS) such as tremors and dyskinesia similar to those seen in people with Parkinson's disease[84]. Adjunctive anti-cholinergic agents are often used to combat EPS, due to the interaction between the dopaminergic and cholinergic systems in the basal ganglia, particularly the VTA[85]. The non-specific muscarinic antagonist procyclidine, however, exacerbated positive symptoms in patients with schizophrenia treated

with the antipsychotic flupentixol[86], while the muscarinic M_1 preferring antagonist biperiden worsened positive symptoms and ameliorated the negative symptoms in patients who were otherwise drug free for at least 2 wk[87]. Patients with schizophrenia who were taking benzatropine, a muscarinic antagonist, as an adjunctive therapy had impaired semantic organisation[88]. These studies show that pharmacological interventions affecting the central cholinergic tone in patients with schizophrenia has a complex effect on symptomatology, and that more specific modulation of the system may be beneficial for ameliorating the symptoms of the disorder.

Xanomeline: A proof of principle drug trial in schizophrenia

A small, double-blind, placebo controlled trial of an agonist selective for the muscarinic M_1 and M_4 receptors, xanomeline, was conducted in a cohort of treatment resistant schizophrenia patients[39]. The xanomeline treated group showed significant improvement in both positive and negative symptoms as measured using the positive and negative syndrome scale and the brief psychiatric rating scale as well as improvements in a battery of cognitive tests, particularly in working memory and verbal and visual learning[39]. Unfortunately, peripheral adverse events were observed including vomiting, nausea and gastrointestinal distress similar to those observed in previous Alzheimer's disease trials[89,90]; this led to the discontinuation of xanomeline. This compound highlighted two important points regarding muscarinic receptors as drug targets. Firstly, its efficacy in the schizophrenia trial[39] supports the body of evidence suggesting that muscarinic receptors, particularly muscarinic M_1 and M_4 receptors, are viable targets for treatment of schizophrenia. Secondly, the problem of selectivity for specific muscarinic receptors. High selectivity for individual muscarinic receptors is difficult to achieve with orthosteric ligands, due to the high homology between their orthosteric binding pockets. The side effects observed in the xanomeline trials were considered to be the result of "off target" activation of muscarinic receptors, particularly muscarinic M_2 and M_3 receptors[89,90]. Clearly, there is a need for ligands that are more selective, both for drug development and investigating individual receptors both *in vivo* and *in vitro*; allosteric compounds present a possible solution.

RECEPTOR ALLOSTERISM

An allosteric site is a binding site on a receptor distinct from the orthosteric binding site of the endogenous ligand that can be activated by proteins or small molecules to elicit a biologic response (allosteric agonist) or modulate the response of an endogenous molecule, orthosteric agonist or orthosteric antagonist biding to the orthosteric site (allosteric modulator). Allosteric modulators increase [positive allosteric modulator (PAM)] or decrease [negative allosteric modulator (NAM)] the ability of orthosteric ligands to activate or inactivate the receptor. These terms describing allosterism are defined according to the guidelines set out by the International Union of Basic and Clinical Pharmacology[91].

Characterisation of putative M_1 receptor allosteric site

In an attempt to better understand the muscarinic M_1 receptor and aid drug development, site-directed mutagenesis techniques have identified amino acid residues of the muscarinic M_1 receptor implicated in the binding of compounds to the orthosteric site, the allosteric site, and in the functional cooperativity between the orthosteric and allosteric sites. From these studies, the orthosteric site is considered to be a pocket deep in the transmembrane domains (TMs; reviewed in[92]). Recent X-ray crystallographic determination of the structures of the muscarinic M_2[93] and M_3[50] receptors has confirmed the orthosteric site is located in a pocket that forms a channel between the TMs for these receptors. The differential binding specificity of ligands to the different receptors is thought to relate to the structural position of the transmembrane domains relative to one another[49,50].

An early study characterising the allosteric site of the muscarinic M_1 receptor performed alanine scanning of the extracellular loop (ECL) amino acid residues conserved across the five muscarinic receptors. The authors found that the only residue to have any appreciable effect on the binding characteristics of gallamine (NAM) was Tyr400, found in the 3rd ECL, leading them to hypothesise that the allosteric site of gallamine is close in space to the orthosteric site[94]. More recently, other residues that are implicated in binding to the allosteric site (Figure 2A) and functional cooperativity between the allosteric and orthosteric sites (Figure 2B) of the muscarinic M_1 receptor were identified in the 2nd, 3rd, 4th, 6th, and 7th TMs and the 2nd ECL[95,96]. Computer modelling of the residues identified in mutagenesis studies has predicted a putative binding site which is topographically distinct from the orthosteric site, mainly composed of residues from ECL2, TM Ⅱ, and TM Ⅶ[96]. Notably, these studies using 1-(4-methoxybenzyl)-4-oxo-1, 4-dihydroquinoline-3-carboxylic acid (BQCA)[96] and a structural derivative of BQCA (benzoquinazolinone 12)[95] found that mutating the Tyr400 residue completely abolished binding to the allosteric site of the muscarinic M_1 receptor; in agreement with the study using gallamine. Interestingly, the residues implicated in allosteric binding to the muscarinic M_1 receptor are highly conserved across all five muscarinic receptors, while residues implicated in the functional cooperativity between the orthosteric and allosteric sites are not; leading the authors to hypothesise that these residues underlie the muscarinic M_1 receptor specific cooperativity of these ligands[96].

Muscarinic M_1 receptor allosteric compounds

There has been considerable interest in using allosteric ligands to target GPCRs implicated in a variety of disease

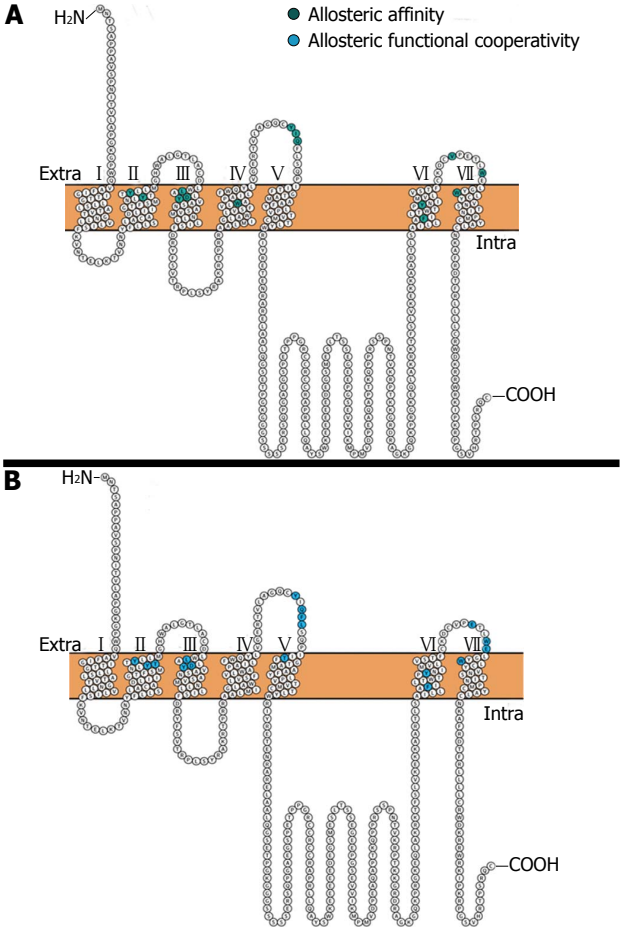

Figure 2 Snake diagram of muscarinic M_1 receptor. The snake diagrams begin with the extracellular amino terminal and terminate at the intracellular carboxyl terminal. Highlighted are amino acids identified by site directed mutagenesis as being implicated in (A) allosteric ligand binding (green) and (B) functional cooperativity (blue)[94-96]. Roman numerals denote transmembrane domains. Diagrams generated using Protter[113]. Extra: Extracellular domain; Intra: Intracellular domain.

states (reviewed in[97,98]). Here we will discuss a number of attempts to develop compounds targeted to the allosteric site of the muscarinic M_1 receptor. Early evidence for allosteric ligands came from the discovery that brucine is a weak PAM[99] and allosteric agonist of muscarinic M_1 receptors[100] demonstrating that potentiation of the muscarinic M_1 receptor is possible. Since this discovery, multiple allosteric agonists of muscarinic M_1 receptors have been identified by high through put screening; including: AC-42[101]; 77-LH-28-1, which increased gamma oscillations, which are associated with memory[56], in rat hippocampal slices and hippocampal cell firing *in vivo*[102]; 1-(1'-2-methylbenzyl)-1,4'-bipiperidin-4-yl)-1H-benzo[d]imidazol-2(3H)-one (TBPB), which reversed amphetamine induced hyper-locomotion in rats[103], a model predictive of antipsychotic-like activity; LuAE51090, which improved mouse performance on the delayed alternation Y-maze[104], a model of working memory; and GSK1034702, which improved episodic memory in humans on the nicotine withdrawal model of cognitive dysfunction[38]. Additionally, [^{11}C]GSK1034702 was clinically trialled as a positron emission tomography (PET) ligand (clinicaltrials.gov identifier: NCT00937846) to assess its blood brain barrier penetrance and distribution *in vivo*; unfortunately [^{11}C]GSK1034702 was deemed unsuitable as a PET ligand for quantification of muscarinic M_1 receptors *in vivo*[105].

Notably, Researchers at Merck Laboratories identified the first highly selective PAM of the muscarinic M_1 receptor: BQCA[106]. BQCA increases ACh affinity for human cloned muscarinic M_1 receptors demonstrating a selective, dose dependant potentiation of ACh's ability to displace [^{3}H]n-methyl scopolamine ([^{3}H]NMS), a pan muscarinic antagonist, binding to muscarinic M_1 receptors expressed in Chinese hamster ovary cells[107]. Further, BQCA has been demonstrated to potentiate muscarinic M_1 receptors cloned from rhesus, dog, mouse and rat muscarinic M_1 receptors as well as selectively potentiate native mouse muscarinic M_1 receptors *in vivo*[107,108]. Recently, we demonstrated that BQCA dose dependently potentiates ACh's ability to displace [^{3}H]NMS in human brain homogenate and that a subset of individuals with schizophrenia with a loss of cortical muscarinic M_1 receptors[78] have a decreased response to BQCA's modulation of ACh binding[109]. Modification of this compound by chemical motif substitution[37,110] is ongoing. A selective muscarinic M_1 receptor PAM based on the BQCA scaffold, 1-{[4-cyano-4-(pyridine-2-yl) piperidin-1-yl] methyl}-4-oxo-4 H-quinolizine-3-carboxylic acid (PQCA)[37], attenuated scopolamine deficits in novel object recognition, self-ordered spatial search, and the object retrieval detour tasks in rats, cynomolgus macaques, and rhesus macaques, respectively[111]. The efficacy of PQCA in these three models of cognitive function further supports the role of the muscarinic M_1 receptor in cognition and demonstrates the potential of this class of compound as useful therapeutics. Additionally, Merck have taken MK-7622, a muscarinic M_1 receptor PAM to phase Ⅱ clinical trial (clinicaltrials.gov identifier: NCT01852110) as an adjunct therapy to an acetylcholinesterase inhibitor (donepezil, rivastigmine or galantamine) for the treatment of cognitive impairment and disease progression in Alzheimer's disease. This highlights the promise of allosteric modulators as pro-cognitive agents.

CONCLUSION

Allosteric modulators of muscarinic M_1 receptors provide a promising method for developing effective, well-tolerated therapies to redress cognitive impairment, particularly in schizophrenia and other diseases characterised by significant cognitive impairment. Historically, treating any disorder with drugs targeting muscarinic receptors has been hampered by the difficulties associated with targeting the orthosteric binding site, particularly the propensity for "off target" side effects, due to the high degree of homology between the orthosteric binding sites of the five muscarinic receptors. Fortunately, the discovery of highly selective allosteric ligands provides a

potential solution to this problem and provides a unique opportunity to maintain physiological firing patterns, unattainable using orthosteric ligands. However, allosteric modulation comes with its own host of idiosyncrasies to be considered when developing ligands targeting this region of the receptors. At the level of preclinical pharmacology, allosteric compounds provide an exciting opportunity to tease out specific downstream signalling pathways, selectively targeting them to achieve highly specific fine-tuning of receptor response. Additionally, by iterative chemical substitutions of the base compound, multiple parameters can be modified to tailor compounds to specific needs, enhancing some aspects of signalling while inhibiting or not affecting others. Although these aspects of allostery provide unique opportunities, they also highlight a need for care when testing compounds and appropriate modelling paradigms, as particular effects could be overlooked or masked by classical drug screening methods. The emergence of allosteric ligands provides us with the exciting opportunity to develop well-tolerated, highly selective therapies with the ability to fine tune distinct pathways addressing subtle pathological changes in complex disease states.

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MINIREVIEWS

Adalimumab and pharmacokinetics: Impact on the clinical prescription for inflammatory bowel disease

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Abstract

Anti-tumor necrosis factor (TNF) drugs are widely prescribed for inflammatory disease. A loss of response to adalimumab is frequent and the pharmacokinetics of anti-TNF therapy have important implications for patient management. Individual factors such as albumin, body weight, and disease severity based on the C-reactive protein level influence drug metabolism. Adalimumab trough levels are associated with clinical remission. On the other hand, the detection of antibodies is associated with clinical relapse. Immunosuppressive therapy could reduce antibody formation although the clinical impact is not proven. New algorithms are available to provide personalized treatment and adapt the dosage. More data are needed on dose de-escalation.

Key words: Pharmacokinetics; Adalimumab; Crohn's disease

Core tip: We have reviewed all the recent data about the factors that influence Adalimumab pharmacokinetics and the impact for the clinicians in the assessment of inflammatory disease. We looked at the inter patient variability, the drug clearance, antibodies detection, the effect of concomitant use of immunosuppressive.

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INTRODUCTION

Crohn's disease (CD) is a chronic disease due to immune dysregulation of the commensal enteric flora in genetically susceptible individuals. There is overproduction of the tumor necrosis factor (TNF) alpha by monocytes

Table 1 Factors influencing Adalimumab response

Sub-cutanous absorption	Age/sex
	Body weight
	Injection site
Disease activity	CRP
Nutritional condition	Albumin

CRP: C-reaction protein.

macrophages and T cells[1-3].

Therapeutic monoclonal antibodies targeting the tumor necrosis alpha pathway for the treatment of immune disease have been shown to be effective. Adalimumab is a recombinant fully human subcutaneously delivered immunoglobulin G1 monoclonal antibody. It has binding properties and affinities for the soluble and transmembrane form of TNF alpha[4]. Anti-TNF drugs have been approved for use in moderate to severe CD when corticosteroids and/or immunomodulators have failed[5].

Twenty-five percent to 30% of patients show no or limited response to these treatments and treatment becomes ineffective during maintenance therapy in 50% of responders[2,6]. Dose escalation is often necessary to maintain a clinical response[7,8]. In addition, the aim of treatment has changed from sustained corticoid free remission to mucosal healing[9]. Little is known about the exposure - response relationship and the factors affecting it. These factors must be clarified to improve the therapeutic efficacy of these drugs. In addition, the pharmacology of anti-TNF drugs depends on the structure of the antibody as well as the properties of the target antigen[2,10]. All of these data suggest that individualize therapy and dosing are necessary[11].

This review will present the most recent data on the factors influencing Adalimumab pharmacokinetics and their clinical impact in the assessment of inflammatory bowel disease.

Pharmacokinetic variability

Maintaining effective concentrations of anti-TNF drugs is not easy and deciding on the appropriate dose depends mainly on clinical symptoms or biological data. Pharmacokinetic data have recently been shown to help with this decision[2].

There seems to be different types of non-responders with several pathogenic mechanisms causing non-response. It is unclear why certain non-responders to one anti-TNF respond to another one while others require a different drug.

One potential explanation for the lack of response to TNF is incomplete suppression of TNF activity[4]. Suboptimal exposure may be due to underdosing, rapid drug clearance and/or the development of anti-drug antibodies.

Inter-patient variability

The effective dose of each individual must be identified to adjust the doses of anti-TNF during the course of drug absorption. For example, subcutaneous absorption varies among patients due to lymphatic drainage, a smaller volume of the drug than with intravenous administration and the risk of immunogenicity associated with a skin reaction[4]. Subcutaneous absorption is slow, incomplete and variable. It takes between 2 to 8 d to reach maximum plasma concentrations. Fifty percent to 100% of the administered dose is absorbed, depending on age, body weight and injection site[2]. Low albumin as well as high body mass index (BMI) and male sex increase drug clearance as shown with Infliximab[12-14]. Adalimumab concentrations were also negatively associated with C-reaction protein (CRP) and correlated to disease severity (Table 1)[15,16]. Disease type has also been shown to influence drug response because patients with ulcerative colitis seemed to have faster clearance than CD patients. One hypothesis is that the overall inflammatory burden in patients with ulcerative colitis is higher with a greater area of mucosal lesions and a greater loss of medication in the intestinal lumen. These data must be confirmed[4].

While inter-individual levels vary, intra-patient adalimumab levels are relatively stable over time (28 wk of follow-up)[16].

Clearance

Because of their high molecular weight, monoclonal antibodies do not undergo renal elimination or metabolism by hepatocytes[4,12].

The primary route of IgG clearance is the intracellular proteolytic catabolism *via* the reticuloendothelial system with receptor mediated endocytosis. This is a saturable route of clearance[2,4].

ANTIDRUG ANTIBODIES

The drug provokes antibody formation. An inactive drug antibody complex (neutralizing antibody), may result in decreased efficacy. The drug antibody complex can also be cleared, providing an alternative clearance pathway for therapeutic protein[17,18].

Drug and antibody dosing can be performed. In clinical practice, Maser *et al*[19] first described the correlation between detectable infliximab trough levels and improved clinical outcomes in CD patients. Results were similar for rheumatoid arthritis[20]. Baert *et al*[21] first reported that patients with anti-infliximab antibodies lost the response to therapy faster than those without. Low infliximab trough levels and high antibodies were significantly more prevalent in patients with a loss of response. Most of the time, the detection of antibodies preceded the clinical loss of response by 2 mo.

Numerous studies have confirmed the positive correlation between adalimumab levels and efficacy and the negative correlation between adalimumab antibodies (ADA) and clinical response[11,22,23].

ADA levels vary from 2.6% to 46% and have increased with the development recent methods measuring free and bound ADA[3,15,22,24-27]. The risk of developing antibodies against a second anti-TNF is increased in patients who

develop antibodies against a first anti-TNF agent and in this case, they usually appear during the first year[28-31].

The notion of transient antibodies has emerged, defined as antibodies that disappear for 2 consecutive infusions. These antibodies, which appear after a median of 13.5 mo, represent 23% of antibodies[15]. Paul *et al*[32] studied 13 patients treated with adalimumab without concomitant therapy. Five patients had transient antibodies with no impact on clinical outcome.

Factors influencing antibody formation have been identified: Low early serum adalimumab concentrations have been shown to increase the future risk of antibody formation[15]. These concentrations have predictive value and could help as a guide to optimize treatment before symptoms develop. Higher post induction concentrations decrease the risk of antibody formation[13,15].

The level of antibodies rather than a simple positive/negative status is also important and the cut off is not known. For Yanai *et al*[33] ADA > 4 g/mL is predictive of failure with a 90% specificity while it is 10 ng/mL for Roblin *et al*[34]. All these factors influence efficacy as well as safety due to hypersensitivity reactions.

However, ADA are not the only mechanism because a loss of response was observed in up to 50% of patients while anti-ADA were detected in 10%-15% of the cases[4].

ADALIMUMAB AND ANTI-ADALIMUMAB ANTIBODY DETECTION

Different methods were developed to measure adalimumab and ADA with some limitations for ADA depending on the detection of non-neutralizing antibodies and of free and/or bound ADA. So the method used influences the interpretation of results.

Enzyme-linked immunosorbent assay (ELISA) is the most common test for measuring adalimumab levels and for ADA detection in patient serum. For drug measurement, TNF coated on the assay plate is exposed to patient serum and the presence of adalimumab is revealed by a labelled polyclonal anti-immunoglobulin (Figure 1). All anti-TNF drugs can be detected in this assay and give a signal which explains why adalimumab can be detectable with this test even if the patient is treated with another anti-TNF.

For ADA detection, the therapeutic antibody [Fab or F(ab)2 fragment] is coated on the assay plate and exposed to patient serum and the presence of ADA is revealed by a labelled therapeutic antibody (Figure 2). However, ELISA has the following limitations: Rheumatoid factors, heterophilic antibodies, both may interfere in antibody detection, ELISA may fail to detect IgG4 antibody which may dominate after prolonged immunization and anti-TNF may aggregate on plastic surfaces giving false positive results. More important ELISA is drug-sensitive and only detects free ADA. ADA cannot be detected in presence of the drug as they are complex with a risk of false negative results. Thus, certain investigators state that ADA results are inconclusive if drug levels are elevated in sera testing negative for ADA because of the presence of antibody-drug complex[35].

Different methods have been developed to overcome this problem. Separate drug-antibody complexes by acid dissociation (pH shifting) were proposed and certain authors found that 20% more ADA could be detected. Indeed the level of ADA is underestimated as they are detected only if the concentration of antibody exceeds that of the drug in the serum[36]. In a variant of this assay, the pH shift anti-idiotype antigen-binding test (PIA) was developed in which rabbit anti-idiotype Fab is added to inhibit re-formation of ADA-drug complexes (PIA)[37]. However by this process, incomplete dissociation, re-formation of complexes or even irreversible destruction of ADA binding epitopes may occur. In another test, the drug was used as a capture antibody and anti-lambda antibody was used as a detecting antibody[38].

Fluid assays were also developed to measure drugs and ADA. In the PIA, immunoglobulins from patient sera were aggregated on a protein (Sepharose) and the presence of ADA was revealed by radiolabelled anti-TNF or F(ab)2 (to avoid rheumatoid factor interference). In the homogeneous mobility-shift assay (HMSA), a fluorescent labelled anti-TNF was used to capture free and bound ADA were separated by size exclusion high performance liquid chromatography (unclear)[39]. However, as complexes could be artificially split during chromatography, non-neutralizing ADA *in vivo* could be detectable by PIA or HMSA with no real clinical relevance.

The last assay was the cell based reporter gene assay (RGA)[40]. This is a functional test based on the detection of TNF activity. It is less sensitive than ELISA and HMSA but highly specific for the clinical response because it detects anti-TNF activity and neutralizing anti-drug antibodies alone thus mimicking the effect of ADA *in vivo*. Steenholdt *et al*[41] recently showed that unlike HMSA and PIA which gives false positives, the results of ELISA and RGA were correlated.

The clinical relevance of low concentrations of ADA that are not detectable in drug sensitive assays has not be clarified and ELISA is actually the most commonly used test because it is easy to perform, less expensive and correlated to the cell assay detecting neutralizing ADA. However, a blood sample should be taken before the next injection and testing should not be performed on blood obtained close to when the drug is administer to be sure to measure ADA without drug interference.

CONCOMITANT USE OF IMMUNOSUPPRESSIVE

Theoretically the effect of concomitant use of immunosuppressive therapy is to reduce antibody formation, to increase the anti-TNF alpha drug in serum and to decrease drug clearance for better clinical outcomes[1].

Two studies have shown that combination therapy minimizes the immunogenicity for Infliximab and Adali-

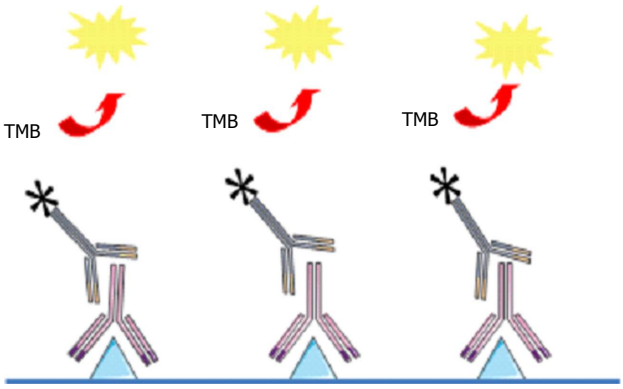

Figure 1 Adalimumab detection. TMB: 3,3',5,5'-Tetramethylbenzidine.

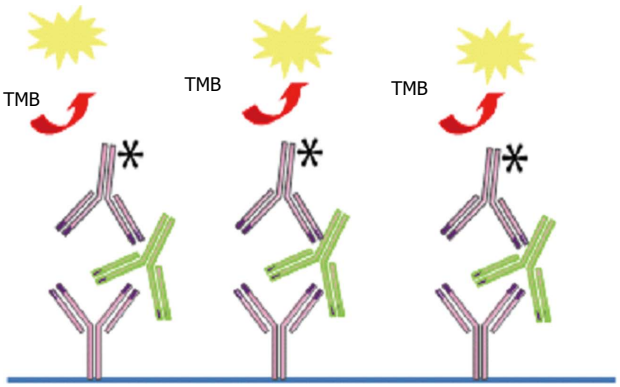

Figure 2 Anti-adalimumab antibodies detection. TMB: 3,3',5,5'-Tetramethylbenzidine.

mumab[42,43]. In Classic 2, 2.6% (n = 7) of 269 patients developed antibodies to Adalimumab and none of the patients with concomitant immunosuppressive therapy had antibodies. Among the 7 patients with antibodies, 43% were in remission 24% and 29% at week 56. A recent study by Baert *et al*[15] confirmed that concomitant use of immunosuppressive therapy prevented antibody formation. In a retrospective analysis of 148 patients, a low serum adalimumab concentration in monotherapy was found to increase the risk of antibody formation. On the contrary, a high post induction drug concentration decreased the risk of antibody formation[15].

However, in their meta-analysis Paul *et al*[3] and Mazor *et al*[22] found that concomitant immunosuppressive therapy did not influence adalimumab levels. In a retrospective study among 217 patients treated with adalimumab, van Schaik *et al*[43] found no beneficial effect of immunosuppressive co-medication for antibody formation. The limit of the study is that they did not evaluate clinical response.

A cut off value for adalimumab levels ranged from 4.8 to 5.9 g/mL whatever the method of measurement for a clinical benefit[3].

However it is important to emphasize that the cut off value depends on the dosing method which is often different in published studies. In addition most of the assays were not compared.

Clinical utility of therapeutic drug monitoring

Long lasting remission of CD can be optimized by maintaining adequate drug levels and preventing antibody formation. Defining predictors of response to anti-TNF alpha and indications for dose escalation will help clinicians choose the best therapy for the appropriate IBD patients with to maximize efficacy and minimize toxicity.

Dose escalation to weekly therapy was needed in 16 (4%) of patients during the 1st year of adalimumab therapy in a prospective study of 201 patients[44]. CRP at week 12 was predictive of clinical efficacy. Azathioprine decreased the probability of dose escalation in this study. In the CHARM trial of 260 patients, 27.3% changed to weekly dosing during the first year and an additional 13.1% changed to weekly dosing during the second year. In another study, 168 patients were followed-up for a median of 20.4 mo. All failed to response to infliximab. Sixty-seven percent responded to Adalimumab at week 12, 65% had to step up to 40 mg per week and 71% responded to the dose escalation. Discontinuation was related to low adalimumab levels and the presence of antibodies[24].

Dose escalation is effective for managing secondary loss of response in CD. One study followed-up 92 patients for 170 wk who achieved a primary response. Eighty percent had clinical response after dose escalation and 56% experienced tertiary loss of response[45]. For ulcerative colitis the rate of escalation was 38.4% for early non responders. At week 52, 25% were in remission[46]. A high body mass index (BMI) and non-response to infliximab were predictive for a dose escalation[7]. There are no pharmacologic data in these studies.

Pharmacokinetics can help select patients who will benefit from dose escalation. A study on mucosal healing has confirmed the association between trough adalimumab levels, clinical remission and mucosal healing[47].

Roblin *et al*[47] explored the clinical utility of therapeutic drug monitoring of Adalimumab in a prospective observational study. The cut off for adalimumab blood levels was 4.9 g/mL. They demonstrated that patients with low blood levels and no antibodies had a better response than patients with antibodies whatever the blood level of drug. The presence of antibodies was related to nonresponse to adalimumab.

The induction phase of treatment also influences antibody formation: Baert *et al*[15] showed that low early drug concentration after induction influenced the risk of antibody formation. These findings show the predictive value of measuring serum adalimumab concentrations early to guide treatment optimization before antibody formation and symptom occurrence. Patients with more severe inflammation require higher than average drug doses to obtain the necessary degree of drug exposure and optimum results.

The reason for discontinuation of infliximab was a clear clinical predictor of response to adalimumab. Among the primary non-responders to infliximab, short-term response to adalimumab was 36% compared to

83% in those who discontinued infliximab for other reasons. There is a clear relationship between serum drug concentrations, clinical effect and long term efficacy.

Mucosal healing has emerged as a major therapeutic goal in IBD. Roblin *et al*[47] studied the association between therapeutic drug monitoring of ADA and mucosal healing among 40 patients. They have shown that therapeutic drug monitoring of ADA was associated with clinical remission in IBD patients and the negative impact of immunogenicity. Median ADA trough levels were significantly higher in patients who achieved mucosal healing. The optimal cut-off value of ADA trough levels for predicting mucosal healing was generally similar to that observed for clinical remission.

Few studies have explored anti-TNF dosage reduction[48]. Baert *et al*[49] recently explored reduction in IFX dosage. For adalimumab, dose de-escacalation to every 2 wk after successful escalation was possible in 63% of patients. There are no pharmacokinetics data in this study.

One study evaluated Adalimumab doses in 6 patients treated after surgery for CD. Adalimumab trough levels in patients with clinical or endoscopic levels were lower than in those in clinical remission after a 2 years of follow-up[50]. More studies are needed to confirm these data.

CONCLUSION

Anti-TNF drugs are extensively prescribed for inflammatory diseases. Loss of response to adalimumab is frequent and the pharmacokinetics of anti-TNF therapy has important implications for patient management. The pharmacology of adalimumab is not completely understood in particular drug clearance. Individual factors such as albumin, body weight and CRP level also influence drug metabolism. Recent data have shown that there is a clinical benefit to the drug and antibody dosage for patient management. Adalimumab levels are associated with clinical remission. On the other hand, the detection of antibodies is associated with treatment failure. There is also a non-anti-TNF pathway in some patients with treatment failure and another therapeutic should then be proposed. New algorithms are available to provide personal treatment and dose adaptations. More data are needed for dose de-escalation. Monitoring drug levels and optimization of treatment without clinical relapse has not been confirmed in clinical practice.

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MINIREVIEWS

Long-term potentiation in autonomic ganglia: Potential role in cardiovascular disorders

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Abstract

Ganglionic long-term potentiation (gLTP) is an activity-dependent, enduring enhancement of ganglionic transmission. This phenomenon may be induced in autonomic ganglia of an organism under certain conditions where repetitive impulses surge from the central nervous system (CNS) to the periphery. Chronic stress, repetitive epileptic seizure or chronic use of CNS stimulants could induce gLTP, which would result in a long lasting heightening of sympathetic tone to the cardiovascular system causing hypertension and disturbed cardiac rhythm that may lead to sudden cardiac death. These conditions are briefly reviewed in this article.

Key words: Electrophysiology; Epilepsy; Ganglionic long-term potentiation; Sudden unexpected death in epilepsy; Central nervous system stimulants; Sudden cardiac death

Core tip: Heightened activity of the central nervous system (CNS) caused by epilepsy, chronic stress and CNS stimulants could provide strong preganglionic stimulation of autonomic ganglia, which may trigger expression of ganglionic long-term potentiation (gLTP). Expression of gLTP can result in cardiovascular dysfunction that may lead to morbidity and even mortality.

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INTRODUCTION

Before Bliss and Lomo[1] coined the term "long-term potentiation (LTP)" in the hippocampus to describe activity-dependent long-lasting potentiation, similar activity-induced enhancement of synaptic transmission was described in the mammalian sympathetic ganglia[2,3]. However, it was nearly two decades before ganglionic LTP

Table 1 Summery of studies reporting ganglionic long-term potentiation of the nicotinic pathway in various animal species

Animal species	Specific ganglia	Ref.
Rat	Superior cervical ganglion	Brown and McAfee[4] Briggs and McAfee[6] Alkadhi *et al*[15] Alzoubi *et al*[25] Alkadhi *et al*[28] Alkadhi *et al*[29] Alkadhi and Alzoubi[36] Alzoubi *et al*[30]
Cat	Superior cervical, lumbar and stellate ganglia	Alonso-deFlorida *et al*[7] Bachoo and Polosa[8]
Guinea pig	Superior cervical ganglion	Weinreich *et al*[9]
Chick	Parasympathetic ciliary ganglion	Scott and Bennett[14]
Bullfrog	Sympathetic ganglia	Koyano *et al*[11] Kumamoto and Kuba[13] Minota *et al*[10]

Adapted from Alkadhi K, Alzoubi K. In: Sudden Death in Epilepsy: Forensic and Clinical Issues (Chapter 26). CRC Press, 2011: 395-426.

Table 2 Effects of various 5-HT$_3$ receptor agonists and antagonists on compound action potential during ganglionic long-term potentiation induced *in vitro* by high frequency repetitive stimulation

Serotonergic drugs	Mode of action	Compound AP
Serotonin (10-20 μmol/L)	Agonist	Increased
Fluoxetine (10 μmol/L)	SSRI	Increased
m-CPBG (1 μmol/L)	Receptor agonist	Increased
Tropisetron (5 μmol/L)	Receptor antagonist	Reduced
Ondansetron (5 μmol/L)	Receptor antagonist	Reduced
MDL 72222 (0.5 μmol/L)	Receptor antagonist	Reduced
Reserpine pretreatment (3 mg/kg)	5-HT$_3$ depletion	No gLTP
m-CPBG (1 μmol/L) + reserpine	Receptor agonist	Increased

The same drugs produced no significant effect on basal synaptic transmission in control ganglia (adapted from ref. [15,25,29,30,32,33,36]). SSRI: Selective serotonin reuptake inhibitor; AP: Action potential.

(gLTP) was characterized in mammalian and amphibian sympathetic ganglia[4-13] as well as avian parasympathetic ciliary ganglion[14] (Table 1). Later, my laboratory identified serotonin as the neurotransmitter necessary for induction and maintenance of gLTP in the rat superior cervical ganglion[15].

The expression of gLTP is due to a series of events resulting from both the postsynaptic and presynaptic regions, and including activation of enzymes, modulators and second messengers. Whereas LTP of the central nervous system (CNS) is regarded as a cellular mechanism of memory; the function of gLTP is uncertain. It is clear that hyperactivity of the CNS, as in the case of chronic stress or recurrent epileptic seizures, may provide the high frequency stimulation (HFS) necessary to induce the expression of LTP in autonomic ganglia, which may cause deleterious alterations in the cardiovascular system function.

gLTP is induced by repetitive HFS (20 Hz) of preganglionic nerve. Upon cessation of HFS of the preganglionic nerve of rat superior cervical ganglion, test stimuli (0.017 Hz) evoke initial highly potentiated ganglionic responses (compound action potentials), lasting up to 4 min, called post-tetanic potentiation[15-17]. This is followed by steady lesser-potentiated action potentials lasting up to 3 h, indicating an increase in synaptic strength[15,18-20].

Published work from this laboratory determined that initiation of gLTP entails both HFS of the preganglionic nerve and stimulation of 5-HT$_3$ receptors by serotonin originating from certain cells within the superior cervical ganglion of rat[15]. Activation of 5-HT$_3$ receptors is necessary for both initiation and expression of gLTP[15]. Extracellular recording revealed that, in ganglia that have expressed gLTP, serotonin 5-HT$_3$ receptor agonists and blockers, in concentrations comparable to pharmacological doses in clinical settings, have profound effects on the magnitude of gLTP[15] (Table 2), even though the same agents produced no significant effect on basal transmission in ganglia from control rat[15]. Thus, we have established gLTP as the first serotonin-dependent LTP ever reported in a mammalian species[21,22].

The 5-HT$_3$ receptor is a ligand-gated receptor-channel complex, and a member of the superfamily that also includes the nACh receptor[23]. It is known that activation of the presynaptic 5-HT$_3$ receptor causes upsurges in calcium concentration inside rat brain nerve terminals[24]. The role of 5-HT$_3$ receptor in the induction and maintenance of gLTP is presently unclear. Perhaps the activation of 5-HT$_3$ channel-receptor complex at the nerve terminals in ganglia causes localized entry of calcium ions increasing its intracellular concentration to a level adequate for activation of downstream signaling molecules, including protein kinase C (PKC), calmodulin and calcium-calmodulin kinase Ⅱ (CaMKⅡ), which are essential for expressing gLTP[25].

IN VITRO INDUCTION OF GLTP

gLTP can be induced by HFS (20 Hz for 20 s) of the preganglionic sympathetic nerve. This frequency is within the maximum range of *in vivo* firing frequency of preganglionic neurons[26]. The response may be measured *in vitro* by intracellular or extracellular recording techniques[6]. Furthermore, gLTP has been evoked and recorded *in situ* from ganglia of anesthetized animals[7,8,27].

The LTP of the hippocampal CA1 region and gLTP are similar in various aspects. For example, both are saturable in that when fully expressed, another HFS will not cause additional augmentation of synaptic trans-

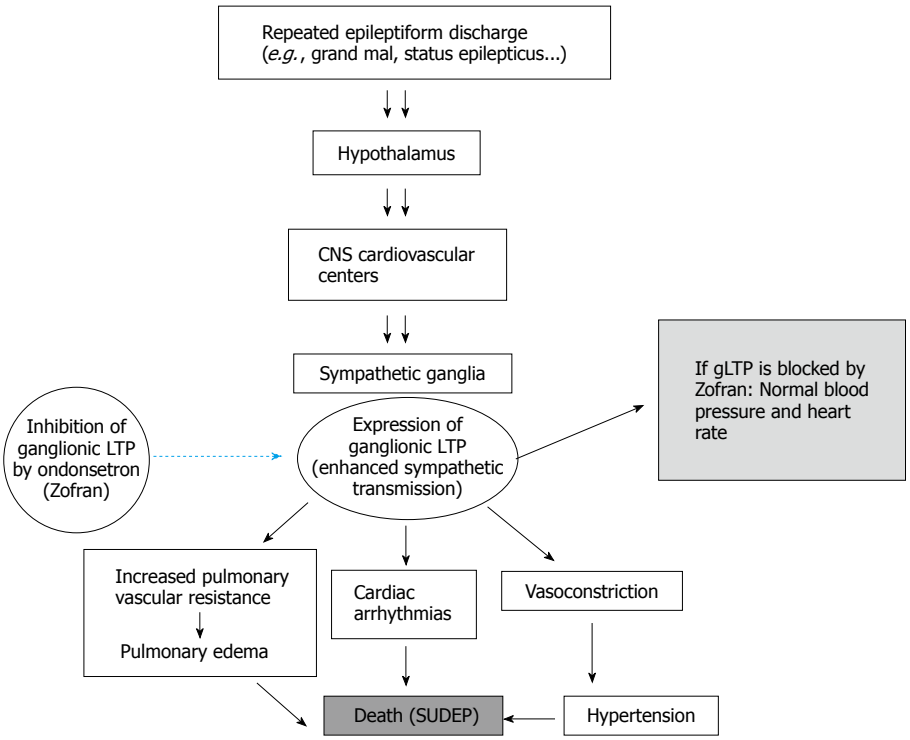

Figure 1 The hypothesis: In the whole animal, epileptic seizures provide the repetitive stimulation required for the expression of ganglionic long-term potentiation in sympathetic ganglia, resulting in increased peripheral resistance, hypertension and cardiopulmonary dysregulation leading to sudden death. Blocking serotonin 5-HT$_3$ receptor with antagonist (Zofran©) in ganglia has been shown to obviate the effect of gLTP. gLTP: Ganglionic long-term potentiation; CNS: Central nervous system; SUDEP: Sudden unexpected death in epilepsy.

mission[28]. Experiments in rat sympathetic ganglia suggest similar molecular mechanisms for the expression of gLTP and hippocampal LTP[29]. Both require a ligand-gated ion channel; here is where hippocampal LTP and gLTP differ: Whereas area CA1 hippocampal LTP requires activation of glutamate NMDA receptor, gLTP requires activation of serotonin 5-HT$_3$ receptor. Similar to NMDA receptor, 5-HT$_3$ receptor is very permeable to Ca^{2+}, which is exceedingly important for launching the molecular cascades responsible for expression of LTP. Strong evidence from this laboratory reveals the involvement of a variety of signaling molecules (*e.g.,* CaMKⅡ, PKC, calmodulin, calcineurin, *etc.*) in the expression of both hippocampal LTP and gLTP[30,31].

The involvement of endogenous serotonin is indicated by absence of HFS-induced gLTP in ganglia of animals treated with reserpine (3 mg/kg) to remove serotonin. However, when these ganglia were treated with serotonin or m-CPBG (a 5-HT$_3$-receptor agonist), HFS invariably induced expression of gLTP (Table 2)[15].

IN VIVO EXPRESSION OF GLTP

An expected outcome from *in vivo* manifestation of gLTP in ganglia is a long-lasting enhancement of sympathetic tone that outflows to the cardiovascular system. Work from this laboratory has established the consequences of *in vivo* induction of gLTP in sympathetic ganglia on blood pressure[29,32,33]. We hypothesize that CNS repetitive activity causes similar outflow to preganglionic nerves, which together with endogenous serotonin may trigger expression of gLTP of sympathetic ganglia. Expression of gLTP produces prolonged and steady increase of sympathetic tone to the cardiovascular resulting in hypertension and disturbed cardiac rhythm (Figure 1).

We hypothesized that chronic psychosocial stress can induce *in vivo* expression of gLTP in sympathetic ganglia, which results in a constant rise in sympathetic tone thus contributing to or initiating a rise of blood pressure. We tested this hypothesis in four animal models of hypertension; aged rats, spontaneously hypertensive rat (SHR), obese Zucker rat and the psychosocial stress model[28,33-35]. We investigated the existence of gLTP in ganglia from these models. For example, in psychosocially stressed hypertensive rats, treatment with tropisetron (ICS; a 5-HT$_3$ receptor antagonist) resulted in normalizing blood pressure of these rats (Figure 2; ref. [29]). Parallel outcomes were obtained in SHR and obese Zucker rat[29,32]. This strongly indicated that the hypertension seen in these animals was, at least partly, due to expression of gLTP.

To further ascertain the existence of gLTP in ganglia isolated from these animal models we showed that "basal" transmission in these ganglia was markedly potentiated (Figure 3A) and that this potentiation was blocked when ganglia were treated with 5-HT$_3$ receptor antagonists[29,32,33,35] (Figure 3B). In another series of experiments, we hypothesized that *in vitro* HFS will not induce gLTP in ganglia isolated from hypertensive old rats if, in fact, gLTP has been expressed already in these ganglia *in vivo*. Whereas HFS produced strong gLTP in ganglia isolated from normotensive adult rats, no gLTP was seen in ganglia from old rats[28,35] (Figure 3C). It is worthy to note that in these series, to ascertain the specificity of 5-HT$_3$ receptor we used three different selective antagonists; bemesetron, tropisetron and ondansetron (Zo-

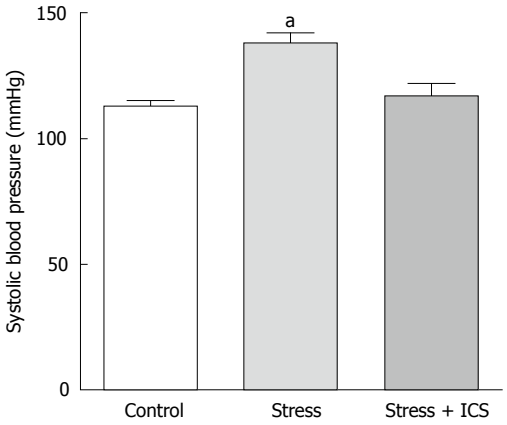

Figure 2 Tropisetron (ICS) normalizes established stress hypertension in psychosocially stressed male rats without affecting blood pressure in control (unstressed) rats measured at day 30 of continuous stress. Blood pressure was measured by tail-cuff plethysmography. Similar results were obtained from female rats. Each point in each group is the mean ± SD from 5 male or female rats. (a) indicates significant difference from other groups (Adapted from ref. [29]).

fran), all were equally effective in blocking gLTP[28,33-35].

POTENTIAL INDUCTION OF GLTP IN BRAIN ILLNESSES AND BY DRUGS

Any procedure that can induce continuous intense flow of impulses from the brain to autonomic ganglia could cause a sustained increase of sympathetic tone to the cardiovascular system, may lead to or contribute to disorders of the system[28-30,32,33,36] (Figure 1). The expression of gLTP in autonomic ganglia that may cause hypertension and cardiac arrhythmias can be a serious risk factor for morbidity and mortality. Evidence that associates expression of gLTP with hypertension has been determined for chronic psychosocial stress[28-30,32,33,36]. Other possible inducers of gLTP are discussed in the following sections.

POSTTRAUMATIC STRESS DISORDER

This serious type of stress results from experiencing harsh distressing occurrences for example witnessing injuries or death, exposure to natural disasters, or experiencing a life-threatening accident. Posttraumatic stress disorder (PTSD) is an incapacitating and potentially chronic disorder characterized by substantial illness. Although similar in some features to chronic stress, PTSD has distinctive pathology[37]. In the first few years following the traumatic event, some PTSD patients may recover, but up to 40% remain chronically symptomatic for years[38]. The major brain areas implicated in the manifestation of PTSD are the prefrontal cortex, amygdala, and hippocampus[39]. During a traumatic event the amygdala sends intensifying impulses to various areas of the brain including prefrontal cortex, hypothalamus, hippocampus and brain stem nuclei. During the course of PTSD, intensified brain activity has been described. For example, PTSD patients showed augmented spontaneous activity in the amygdala and frontal cortex[40]. Moreover, PTSD is linked to increased sympathetic activity represented by elevated blood pressure increased heart rate, and/or increased adrenergic transmitter release[41]. This increase in sympathetic activity could be due to expression of gLTP in autonomic ganglia. However, whether gLTP is present in ganglia during the progression of PTSD and whether cardiovascular disorders are due to gLTP in autonomic ganglia remain to be explored in animal models of PTSD.

EPILEPSY

The excessive and abnormal cortical brain activity in epilepsy is transmitted through the brain stem to the rest of the body and can usually cause various types of seizures. Strong stimulation of the sympathetic nervous system often accompany seizures and can cause hypertension, dispersed injury of myocytes, and increased predisposition to ventricular arrhythmias[42,43]. Several areas in the brain are involved in the cardiovascular effect of epileptic seizures such as the hypothalamus and medulla oblongata, particularly nuclei of the nucleus tractus solitarius, and area postrema, which are closely engaged in regulation of cardiovascular function[44-46]. Therefore, enhanced activity of these areas is communicated to autonomic ganglia and may provide the required repetitive activity that triggers the expression of gLTP in these ganglia. The expression of gLTP results in long-term enhancement of sympathetic tone to the cardiovascular system, causing hypertension and neurogenic cardiac arrhythmias, which can be major risk factors for sudden unexpected death in epilepsy, a dangerous clinical difficulty for certain epileptic patients, especially those with chronic, inadequately controlled seizures.

NICOTINE

Epileptiform brain activity was recorded in the brains of young rats treated with nicotine[47]. In humans, chronic use of tobacco products is known to cause enhanced cholinergic activity in the brain[48-50]. Nicotine can also augment peripheral sympathetic activity through activation of postganglionic nicotinic acetylcholine receptors[51,52]. Moreover, nicotine can release epinephrine from the adrenal medulla into the blood[53,54]. Thus, nicotine causes stimulation of the cardiovascular system that increases heart rate and causes hypertension by action on both peripheral and central sites[53]. Hence, since epileptic patients are more likely to be chronic tobacco users[55], such chronic use of nicotine may result in the expression of gLTP in ganglia or enhancement of the impacts of already expressed gLTP in epileptic tobacco users, thus intensify the risk for cardiovascular dysfunction that may cause sudden death[56].

CAFFEINE

Caffeine, a competitive inhibitor of adenosine receptors,

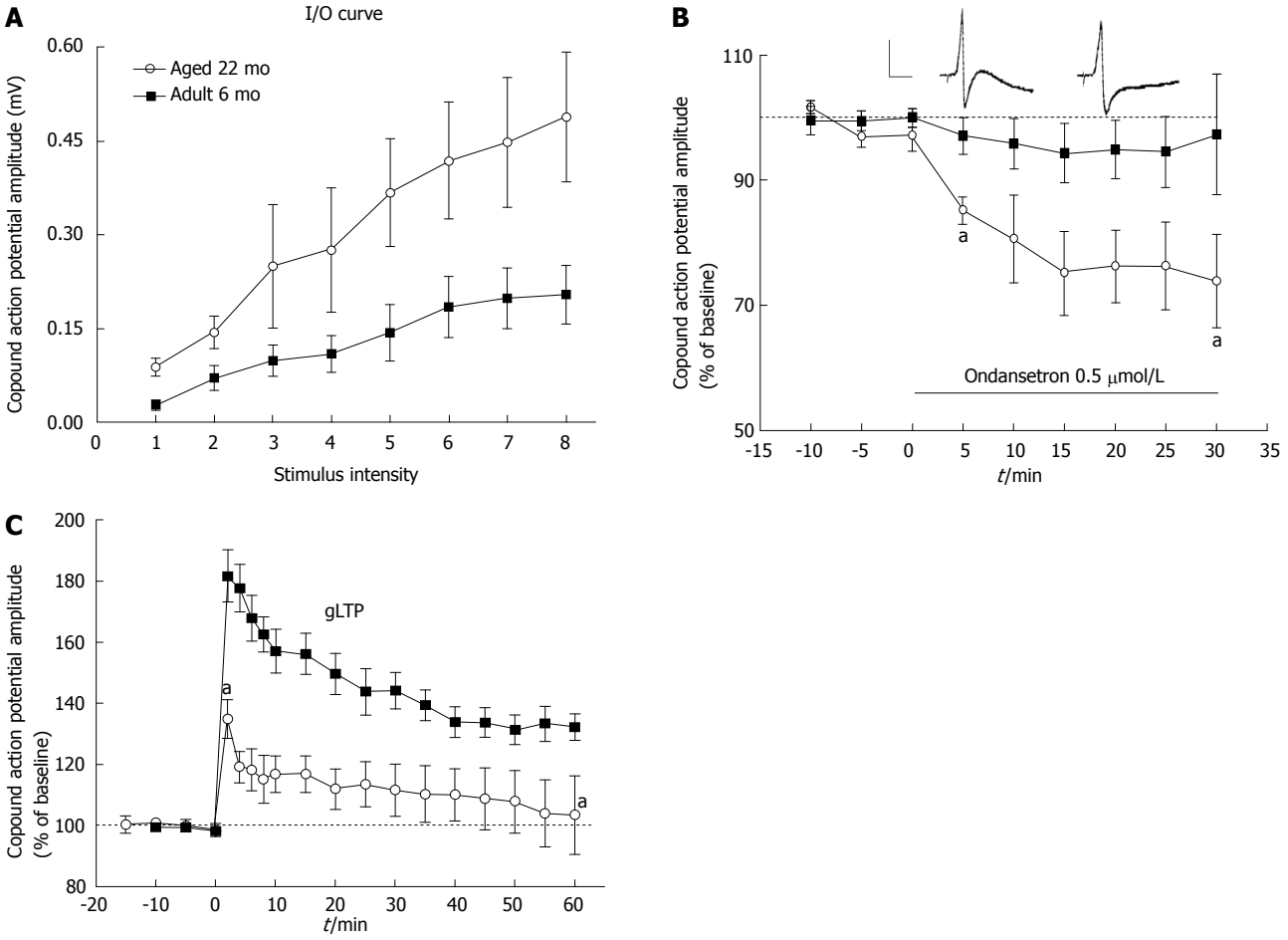

Figure 3 Expression of ganglionic long-term potentiation in sympathetic superior cervical ganglia in aged (22 mo) hypertensive rats. A: Input/output curve (I/O) of aged animal ganglia compared to adult (6 mo) ganglia, indicating enhanced synaptic activity in the aged animal ganglia. Stimulus intensity numbers along the X-axis are arbitrary values, where 1 is the minimal and 8 the maximal response (CAP amplitude in mV). Each point from aged rats is significantly different from matching points of adult rats, and is the mean ± SEM from 5-7 ganglia; B: Inhibition of "baseline" ganglionic transmission in aged rats by a 5-HT$_3$ receptor antagonist ondansetron (Zofran) as an indication of expression of gLTP *in vivo*. Zofran (0.5 μmol/L, solid horizontal line) decreased CAP baseline of ganglia isolated from aged but not of those isolated from adult rats. All points between the two "a" are significantly different ($P < 0.05$) from corresponding point for adult rats. Each point in each series is the mean ± SEM from 5-7 ganglia. Inset: CAPs of aged rats before and after application of drug; calibration 0.5 mV/20 ms; C: High frequency stimulation (HFS: 20 Hz/20 s, at 0 time) of the preganglionic nerves evoked robust gLTP in ganglia excised from adult rats. Identical protocol in ganglia from aged rats produced no gLTP. Each point represents the mean ± SEM from 5 ganglia. Adapted from ref. [35]. gLTP: Ganglionic long-term potentiation; CAP: Compound action potential.

is the most extensively used CNS stimulant because it is consumed in a variety of hot and cold drinks, as well as many prescription and over-the-counter medications. Neuroimaging studies reports show that by acting on brain cortex, caffeine enhances attention and mental arousal[57-59]. However, there is no convincing evidence that the usual doses of caffeine increase the risk of heart attack, sudden cardiac death, or disruption of cardiac rhythm. Nonetheless, a new caffeine source are the so called "energy drinks", which contain uncommonly hefty doses of caffeine. Consumption of such energy drinks may lead to platelet and endothelial dysfunction, which can cause myocardial infarction and other cardiovascular disorders in healthy young adults[60-62] (for review see ref. [63]). Through stimulation of the CNS, heavy frequent intake of caffeine-containing drinks may trigger gLTP in sympathetic ganglia, which could be responsible for the reported cardiovascular disturbances. The danger may be even greater when such consumption of large doses of caffeine is coupled with heavy use of tobacco products.

AMPHETAMINES AND COCAINE

The amphetamines work mainly by modifying the catecholamine system in the pleasure center of the brain[64]. They increase levels of major catecholamines such as dopamine and norepinephrine in a dose-dependent manner[65-67]. A case-control study has linked the use of one of the most commonly used CNS stimulant, methylphenidate (Ritalin), to sudden death in children and teenagers[68].

Cocaine inhibits the monoamine reuptake mechanism in central and peripheral sympathetic nerve terminals in humans[69-71] with end effects similar to those seen with amphetamines. This however, may not be the sole CNS effect of cocaine inasmuch as other reuptake inhibitors do not have cocaine-like effects. The abuse of cocaine is correlated with cardiovascular dysfunction including hypertension, ventricular dysrhythmia, acute myocardial

infarction, and left ventricular hypertrophy. Therefore, the chronic use of CNS stimulants such as amphetamine and cocaine may trigger expression of gLTP, which may lead to morbidity and/or sudden death.

CONCLUSION

Abnormal strong brain activity as in epileptic seizures cause intense activation of ganglionic neurons, which can induce gLTP in sympathetic ganglia leading to long-term heightened sympathetic tone to the cardiovascular system with the ensuing rise in blood pressure and disturbed heart rhythm. Abnormal CNS activity can result from severe brain injuries, ongoing psychological stress, epilepsy, and regular abuse of CNS stimulating substances. Even though these disorders can cause disturbances of the function of the cardiovascular system, their possible link to gLTP has not been studied, except in chronic psychosocial stress. Therefore, it is necessary to determine such links in order to develop therapeutic plans to avoid serious consequences such as sudden cardiac death.

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ORIGINAL ARTICLE

Observational Study

Innovate combination of sevoflurane dilution in dimethyl sulfoxide: A stability study by gas chromatography and nuclear magnetic resonance

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Abstract

AIM

To investigate physicochemical stability of sevoflurane

in dimethyl sulfoxide using gas chromatography with a flame ionization detector and nuclear magnetic resonance (NMR).

METHODS

Undiluted sevoflurane, plus dilutions 1:2, 1:5, 1:10, 1:25, and 1:50 in dimethyl sulfoxide were prepared in a vertical laminar flow cabinet class Ⅱ type B and stored at different temperatures (23 ℃, 6 ℃, and -10 ℃) for 45 d. Sterile 1 mL polypropylene amber syringes to minimize light degradation, caps and needles were used. The presence of sevoflurane and its degradation products in the samples was determined by gas chromatography with flame ionization detector (260 ℃, 40 min), and by ^{1}H, ^{19}F, and proton-decoupled ^{19}F nuclear magnetic resonance.

RESULTS

The gas chromatography analysis showed sevoflurane and dimethyl sulfoxide (DMSO) retention times were 2.7 and 13.0 min, respectively. Pure DMSO injection into the column resulted in two additional peaks at 2.1 and 2.8 min. The same sevoflurane peak at 2.7 min was observed in all the dilutions at -10 ℃, 4 ℃ and 25 ℃. The NMR spectra showed signals consistent with the sevoflurane structure in all the dilutions at -10 ℃, 4 ℃ and 25 ℃. In the ^{1}H spectrum, two signals corresponding to the sevoflurane molecule were observed at 5.12 and 4.16 parts per million (ppm[5]). In the ^{19}F-NMR spectrum, two signals were observed at -76.77 ppm and -157.13 ppm. In the ^{19}F NMR CPD, two signals were observed at -76.77 ppm and -157.13 ppm. The first one showed a doublet (JF-F = 3.1 Hz) which integrated by six fluorine nuclei from the hexafluoro-isopropyl group. The second signal was integrated by a fluorine atom and showed a septuplet (JF-F = 3.1 Hz).

CONCLUSION

This study shows that different concentrations of sevoflurane in dimethyl sulfoxide retain their chemical composition after exposure to different temperatures for a period of 45 d.

Key words: Sevoflurane; Dimethyl sulfoxide; Uclear magnetic resonance; Gas chromatography; Skin ulcers; Drug stability

Core tip: Direct topical application of anesthetic sevoflurane has recently shown beneficial properties in the management of chronic vascular ulcers. A more convenient formulation could be obtained using solutions of sevoflurane in a miscible solvent such as dimethyl sulfoxide. However, no study has yet assessed the physicochemical and pharmaceutical stability of these formulations. Different concentrations of sevoflurane in dimethyl sulfoxide were stored over 45 d at -10 ℃, 4 ℃ and 25 ℃ and assayed by gas chromatography with a flame ionization detector and nuclear magnetic resonance, showing that molecular structures remained unaltered after exposure to a range of temperatures.

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INTRODUCTION

Sevoflurane [1,1,1,3,3,3-hexafluoro-2-(fluoromethoxy) propane] is a highly-fluorinated methyl-isopropyl ether-derived molecule. Chemically, it is a sweet-smelling, non-flammable, colorless substance used as an inhaled anesthetic in the induction and maintenance of general anesthesia in adult and pediatric patients[1]. It has been documented that sevoflurane instillation into skin ulcers has a rapid, intense, and durable anesthetic effect. Despite the existence of some favorable safety data, uncertainty exists on the direct effects of topical undiluted sevoflurane due to its high concentration[2-4].

Dimethyl sulfoxide (DMSO, C_2H_6OS) is a polar aprotic solvent able to solubilize sevoflurane. Chemically, it is a low-volatile, transparent, colorless, hygroscopic substance with wide applications as topical pharmaceutical vehicle because of its ability to penetrate biological membranes. It has shown analgesic, healing, oxygen free-radical scavenger, and antimicrobial properties after topical application[5-11].

As both sevoflurane and DMSO have shown usefulness and safety in preliminary studies in topical administration for the treatment of ulcers, we designed a study aimed at assessing the physicochemical stability of several sevoflurane concentrations in DMSO at different temperatures over 45 d. Stability was assessed using gas chromatography with a flame ionization detector (GC-FID) and nuclear magnetic resonance (NMR). To our knowledge, no similar study on the stability of sevoflurane in DMSO has been reported in the literature.

MATERIALS AND METHODS

Sevoflurane's stability was defined as the maintenance of its physicochemical, microbiological, and biopharmaceutical properties within the specified range during its lifespan, under the influence of several ambient factors such as temperature, humidity, and light exposure[12].

Dilution preparation

Sevorane® 100% v/v (AbbVie®, Campoverde di Aprilia, Italy) was used in the preparation of dilutions using DMSO 99% (Fagron Ibérica, Terrassa, Spain) as a

solvent. Six different sevoflurane-in-DMSO dilutions were used: Undiluted sevoflurane, and dilutions 1:2, 1:5, 1:10, 1:25, and 1:50. One milliliter aliquots were prepared as follows: 0.02 mL of sevoflurane and 0.98 mL of DMSO (1:50), 0.04 mL of sevoflurane and 0.96 mL of DMSO (1:25), 0.9 mL of sevoflurane and 0.1 mL of DMSO (1:10), 0.2 mL of sevoflurane and 0.8 mL of DMSO (1:5), and 0.5 mL of sevoflurane and 0.5 mL of DMSO. Dilutions were prepared in a vertical laminar flow cabinet class Ⅱ type B (Telstar® AH-100, Terrassa, Spain), using sterile 1 mL polypropylene amber syringes to minimize light degradation, caps and needles. Fifteen syringes divided into three sets were prepared, each set consisting of five syringes with different sevoflurane concentrations. The required volume of sevoflurane was drawn up into the syringe and the volume was made up to 1 mL with DMSO. Syringes were shaken gently to allow homogenization of the dilution, secured with caps to prevent product volatilization and sealed in opaque and isotherm polystyrene boxes with freeze blocks inside.

Temperature exposure

The first set of syringes was stored in a dry sealed box stored in a refrigerator at 4 ℃ ± 2 ℃. The second set of syringes was stored in a locked cupboard at room temperature 23 ℃ ± 2 ℃. Finally the third set was stored in a freezer at -10 ℃ ± 2 ℃. Temperature was monitored with digital minimum/maximum thermometers.

Physical analysis

All dilutions were examined for changes in color (against white and black backgrounds), viscosity, and formation of precipitates at the time of preparation and at weekly interval.

Microbiological analysis

Fifty microgram aliquots from each solution were plated on Müeller-Hinton agar enriched with 5% sheep blood (Remel®, Lenexa, Kansas, United States), using a spiral plating device (Microbiology International®, Rockville, Maryland, United States). Plates were incubated at 37 ℃, and the number of colonies growing on the plate was counted after 24 h.

GC-FID

Samples were subjected to treatment with anhydrous sodium sulfate to remove any traces of humidity. One gram of sodium sulfate was added to each sample, vortex-mixed for 1 min and then filtered through 45 μm nylon microfilters. One microliter of each sample was then injected into a gas chromatographer model Focus GC® with a capillary column Omegawax® 250 (30 m, 0.25 mm inner diameter, 0.15 μm film thickness) (Supelco®, Bellefonte, Pasadena, United States) coupled with a flame ionization detector (FID) (Thermo Electron®, Cambridge, United Kingdom). The temperature program was set as follows: 1 min at 40 ℃, heating at 10 ℃/min up to 260 ℃, and maintenance at 260 ℃ for 40 min. The injector temperature was 250 ℃ and the detector temperature was 270 ℃. Nitrogen was used as carrier gas at a rate of 1 mL/min and with a split flow of 20:1. Sevorane® and pure DMSO were analyzed separately following the same procedure in order to obtain a reference for the retention time of sevoflurane and DMSO, as well as to detect the presence of any manufacturing-derived impurity prior to the mixing and storage of the dilutions. The method was validated and applied in concordance with European Pharmacopoeia.

^{1}H and ^{19}F NMR

NMR spectra were acquired using a Bruker Avance DRX 300 MHz® spectrometer equipped with a 5 mm single-axis z-gradient quattro nucleus probe (Bruker Biospin GmbH, Rheinstetten, Germany). A capillary filled with hexadeuterated DMSO (DMSO-d6) was inserted into each NMR tube to obtain a lock signal. All NMR experiments were performed at room temperature. ^{1}H-NMR, coupled ^{19}F-NMR, and proton-decoupled ^{19}F-NMR using a Waltz16 composite proton-decoupling pulse sequence (^{19}F-CPD) spectra were obtained. CPD allows the saturation of the proton channel frequency whilst acquiring the fluorine spectra. This halts the heteronuclear ^{19}F-proton coupling and allows ^{19}F-^{19}F homonuclear coupling only, which is used to confirm an observed ^{1}H-^{19}F coupling. Spectral widths were 3000 and 3700 Hz, respectively. Eight scans were accumulated for ^{1}H and thirty-two for ^{19}F, with an acquisition time of 2.73 and 2.00 s, respectively.

RESULTS

Physical and microbiological analysis

No significant physical or microbiological changes were observed at the storage temperatures after 45 d. Neither color or viscosity changes, nor formation of precipitates were observed at any temperature. At 4 ℃, dilutions 1:10, 1:25, and 1:50 preserved their liquid, colorless state, whereas at -10 ℃ only the undiluted sevoflurane and the 1:2 dilution were liquid. No microbial growth was observed after 24 or 48 h cultures in any sample.

Gas chromatography analysis

Sevoflurane and DMSO retention times were 2.7 and 13.0 min, respectively. Pure DMSO injection into the column resulted in two additional peaks at 2.1 and 2.8 min. These were probably due to manufacturing-derived volatile impurities, most likely dimethyl sulphide (starting material) and dimethyl sulphone (by product), the levels of both impurities were 0.08%. The same peaks were found across all the dilutions of sevoflurane kept at different temperatures (Figure 1). The temperature was maintained at 260 ℃ up to 40 min and the chromatogram baseline was amplified in order to search

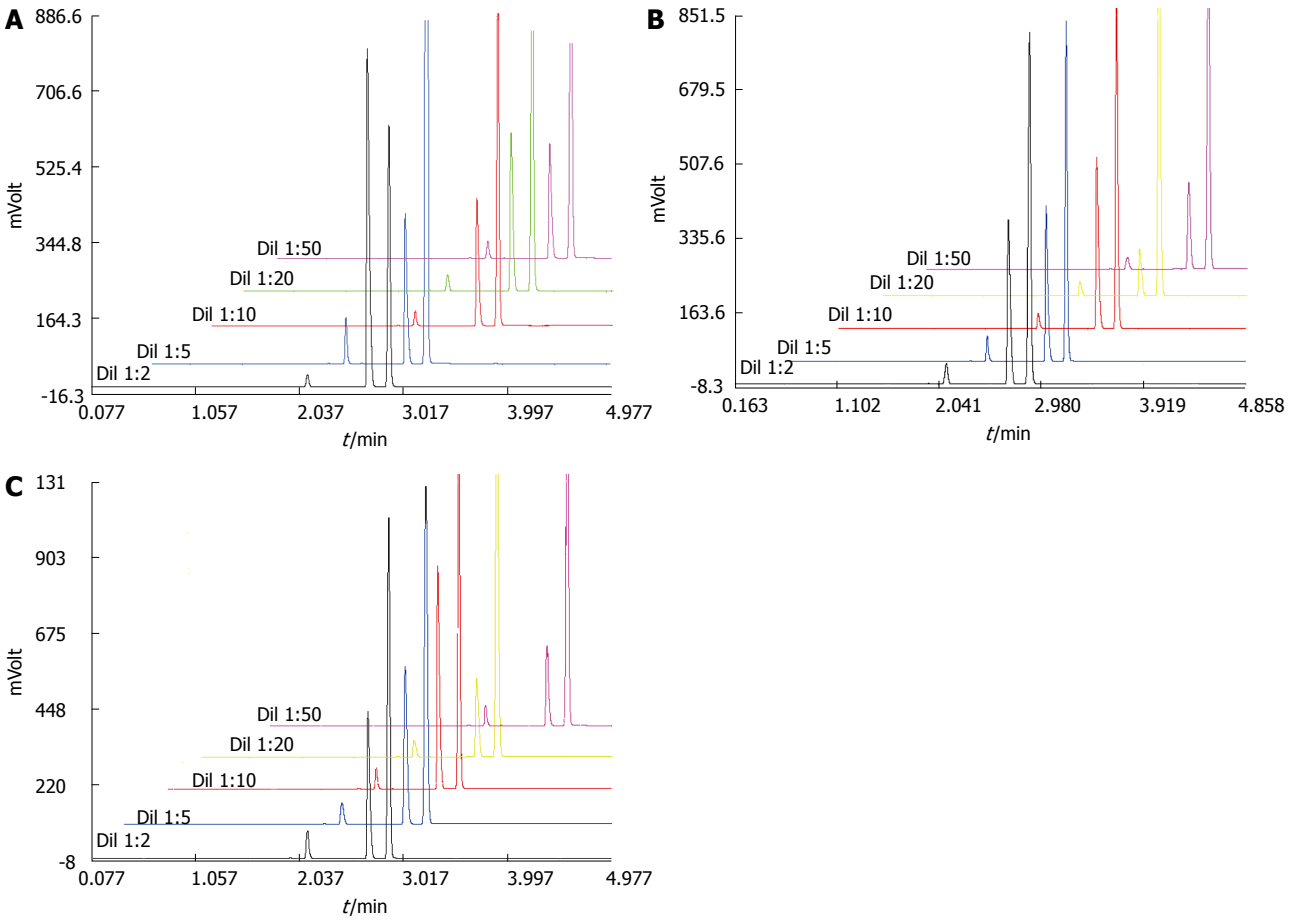


Figure 1 Chromatogram amplification showing the sevoflurane peak at 2.7 min in the different dilutions at -10 ℃ (A), 4 ℃ (B) and 25 ℃ (C).

for possible degradation by-products.

NMR analysis

The ^{1}H, ^{19}F and ^{19}F-CPD NMR reference spectra acquired from undiluted sevoflurane are shown in Figure 2. The ^{1}H and ^{19}F-NMR spectra of all the dilutions kept at different temperatures are represented in Figure 3.

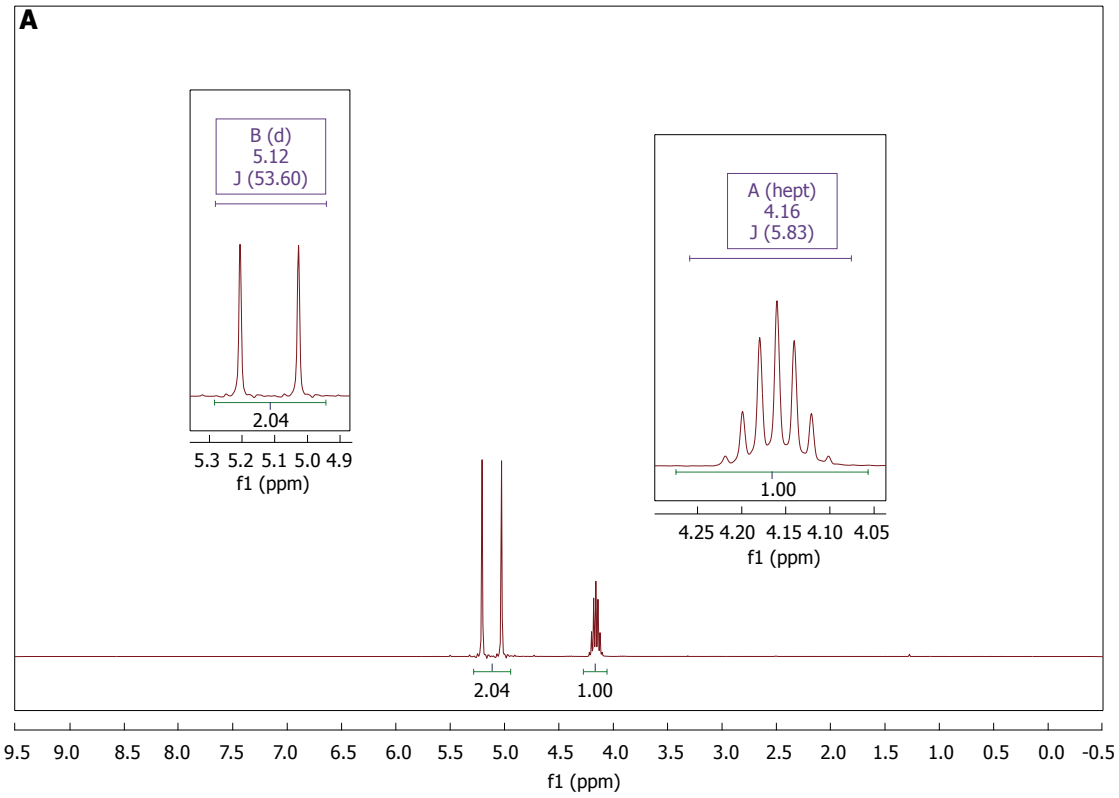
In the ^{1}H spectrum, two signals corresponding to the sevoflurane molecule were observed at 5.12 and 4.16 parts per million (ppm[5]). The signal at 5.12 ppm was integrated by two protons with a doublet multiplicity (coupling constant $J_{H\text{-}F}$ = 53.6 Hz), which was assigned to the protons of the fluoromethoxy group (-OCH_2F). The signal at 4.16 ppm was a septet that integrated one proton (coupling constant $J_{H\text{-}F}$ = 5.8 Hz), which was assigned to the proton from the hexafluoro-isopropyl group $(CF_3)_2CH$-.

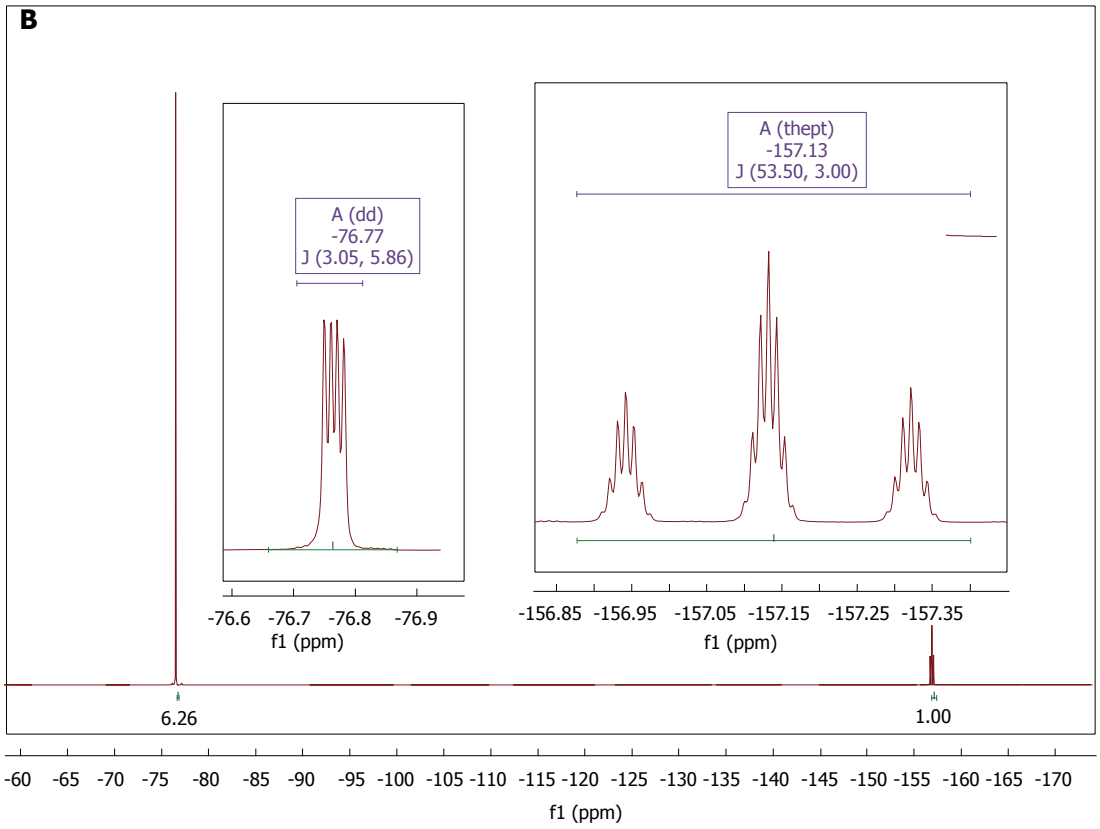
In the ^{19}F-NMR spectrum, two signals were observed at -76.77 ppm and -157.13 ppm. The signal at -76.77 ppm was integrated by six fluorine atoms, consisting of a double-doublet with coupling constants J_1 = 5.9 Hz and J_2 = 3.1 Hz, corresponding to the hexafluoro-isopropyl group $(CF_3)_2CH$-. The multiplicity in this case was due to the coupling of six fluorine atoms with the only proton of the group ($J_{1F\text{-}H}$ = 5.9 Hz); there was a long range coupling with the fluorine atom from the fluoromethoxy group ($J_{2F\text{-}F}$ = 3.1 Hz). The signal at -157.13 ppm integrated for one fluorine nucleus and was a triple-heptuplet with coupling constants J_1 = 53.5 Hz and J_2 = 3 Hz, corresponding to the fluorine atom from the fluoromethoxy group (-OCH_2F). The multiplicity could be explained due to the coupling between the fluorine atom and two adjacent protons ($J_{1F\text{-}H}$ = 53.5Hz), and a long-range five-bond coupling between the fluorine nucleus in the fluoromethoxy group and the $(CF_3)_2CH$- fluorine nuclei ($J_{5F\text{-}F}$ = 3.0 Hz).

In the ^{19}F NMR CPD, two signals were observed at -76.77 ppm and -157.13 ppm. The first one showed a doublet ($J_{F\text{-}F}$ = 3.1 Hz) which integrated by six fluorine nuclei from the hexafluoroisopropyl group. The second signal was integrated by a fluorine atom and showed a septuplet ($J_{F\text{-}F}$ = 3.1 Hz).

DISCUSSION

This study shows that different concentrations of sevoflurane in DMSO (ranging from the undiluted product to a 1:50 dilution) preserve their chemical structure after exposure to a range of temperatures (-10 ℃, 4 ℃, and 25 ℃) over 45 d. No sevoflurane by-products were detected in any of the samples. To our knowledge, the stability of sevoflurane solutions in DMSO has never been


A
B (d)
5.12
J (53.60)
2.04
5.3 5.2 5.1 5.0 4.9
f1 (ppm)
A (hept)
4.16
J (5.83)
1.00
4.25 4.20 4.15 4.10 4.05
f1 (ppm)
2.04
1.00
9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0 -0.5
f1 (ppm)


B
A (dd)
-76.77
J (3.05, 5.86)
-76.6 -76.7 -76.8 -76.9
f1 (ppm)
A (thept)
-157.13
J (53.50, 3.00)
-156.85 -156.95 -157.05 -157.15 -157.25 -157.35
f1 (ppm)
6.26
1.00
-60 -65 -70 -75 -80 -85 -90 -95 -100 -105 -110 -115 -120 -125 -130 -135 -140 -145 -150 -155 -160 -165 -170
f1 (ppm)

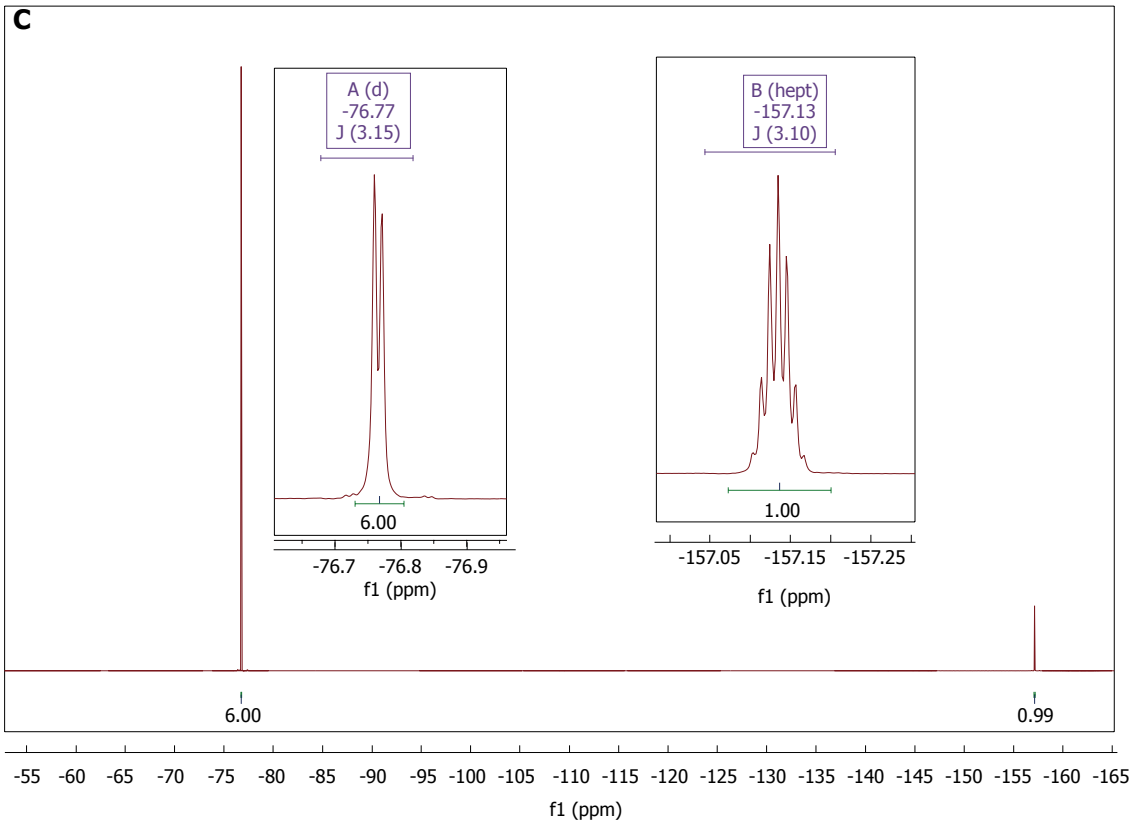


Figure 2 Undiluted sevoflurane nuclear magnetic resonance spectra. A: ^{1}H-NMR; B: Coupled ^{19}F-NMR; C: Proton-decoupled ^{19}F-NMR using a Waltz16 CPD pulse sequence. NMR: Nuclear magnetic resonance; CPD: Composite proton-decoupling.

reported in the literature.

Sevoflurane is a poly-fluorinated methyl-isopropyl ether-derivative commonly used in the induction and maintenance of general anesthesia in both adult and pediatric patients[1]. Recent evidence suggests it might aid healing and mitigate pain associated with vascular ulcers[2-4]. The selection of DMSO as a vehicle for sevoflurane responds to both pharmaceutical and pharmacological needs: It is a polar solvent chemically compatible with sevoflurane over a wide range of concentrations. Additionally, some studies suggest it might possess some analgesic, hydroxyl free-radical scavenger, healing, and antimicrobial properties after topical application[7-11].

Amber polypropylene syringes and caps were used for the preparation and conservation of the aliquots. This responds to the known reactivity between sevoflurane and glass surfaces, which leads to the production of hydrofluoric acid (HF). According to this, an increase in HF concentration would be directly related to the degree of degradation of sevoflurane[13,14].

In this study, two main analytical techniques were used to assess the integrity and physicochemical stability of sevoflurane dilutions: GC-FID and NMR. GC-FID constitutes the gold-standard for the analysis of volatile substances such as sevoflurane[15,16]. This technique combines a high analytical sensibility in the range of parts per billion with a universal response to any compound suffering ionization in the hydrogen flame and with its quantitative capabilities, having a long linear range and low signal-to-noise ratio. NMR is based on the measure of the resonance frequency of active nuclei with spin different from zero (such as ^{1}H and ^{19}F) of a molecule in the presence of a high magnetic field. Given that sevoflurane contains seven fluorine atoms in its structure, combined NMR measuring of ^{19}F and ^{1}H nuclei is an ideal option for the characterization of both sevoflurane and any eventual by-product[17-21]. In the case of finding such breakdown compounds, it would be feasible to quantify the amount of degradation by any of the two techniques, preferably by GC. In addition, GC and NMR are analytical techniques recommended by the European and the American Pharmacopoeias, as well as by the International Conference on Harmonization guidelines (ICH) to study the chemical stability of drugs[22-24]. In the GC analysis, the chromatogram of each sample was compared with those of the reference commercial sevoflurane and the DMSO used. The levels of both impurities observed, dimethyl sulphide (starting material) and dimethyl sulphone (by product), were 0.08%, DMSO impurities are controlled in the PhE at 0.1%[25]. No additional peaks other than those previously present were detected even after a chromatographic run of 40 min raising the oven temperature to 260 ℃. The chromatogram baseline was amplified and searched for such impurities and the results were consistent in all the samples with the absence of degradation.

In the NMR study, sevoflurane signals were observed

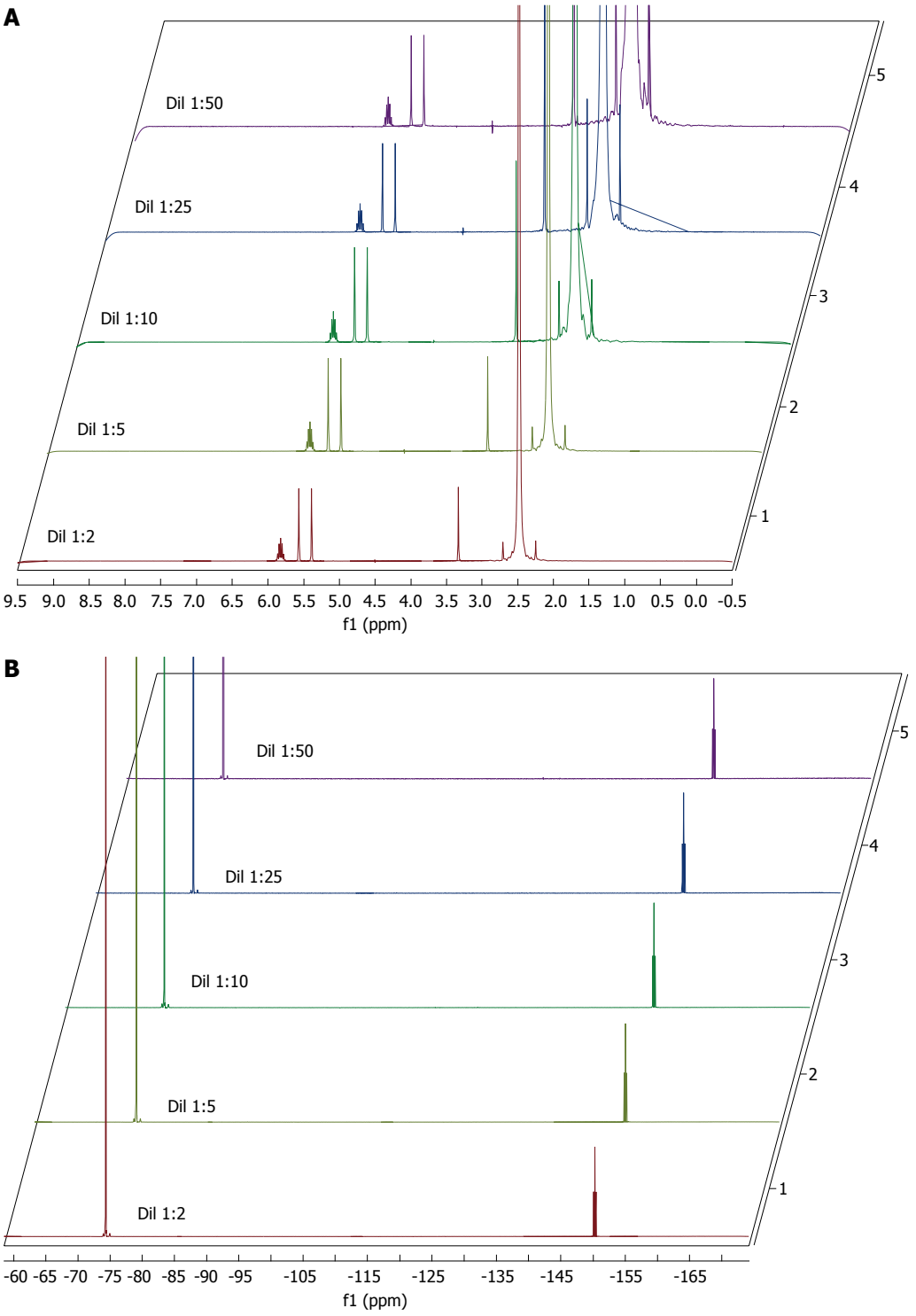


Figure 3 ^{1}H-nuclear magnetic resonance (A) and ^{19}F-nuclear magnetic resonance (B) of solutions kept at all temperatures. The images have been adjusted and sevoflurane peaks assigned the same height so that dimethyl sulfoxide and water signals in the ^{1}H spectrum increase inversely to concentration.

in both ^{1}H and ^{19}F spectra, regardless of the temperature conditions. This confirms the structural integrity of the sevoflurane molecule at a range of temperatures and concentrations, including the undiluted product, as well as the absence of other sevoflurane breakdown products.

Limitations

Caveats associated with these findings rely on the need for longer-term stability assays in order to evaluate the effects of temperature on sevoflurane at different times. Additionally, the effects of light exposure were not assessed.

In conclusion, this study confirms that the chemical structure of sevoflurane structure remains stable both diluted in DMSO at different concentrations and in pure state when subjected to a range of temperatures longer

than a month in polypropylene syringes. These findings warrant further investigation, particularly in light of its potential applications in the management of pain and healing associated to skin ulcers.

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COMMENTS

Background

Direct topical application of anesthetic sevoflurane has recently shown beneficial properties in the management of chronic vascular ulcers. A more convenient formulation could be obtained using solutions of sevoflurane in a miscible solvent such as dimethyl sulfoxide. However, no study has yet assessed the physicochemical and pharmaceutical stability of these formulations.

Research frontiers

The treatment of skin ulcer and structural drug analysis by nuclear magnetic resonance has progressed immensely in recent years. Assessing the physicochemical stability of sevoflurane in dimethyl sulfoxide (DMSO) solutions becomes essential in view of the potential therapeutic applications of such solutions in the management of vascular ulcers.

Innovations and breakthroughs

The present study shows that different sevoflurane in DMSO solutions are stable from a physicochemical and pharmaceutical point of view at a range of temperatures for up to 45 d. This has important implications in the formulation of new pharmaceutical forms aimed at improving the management of vascular ulcers.

Applications

The data in this study suggested that both drugs can be preloaded in polypropylene syringes and stored at different temperatures for the topical management of chronic vascular ulcers.

Terminology

Vascular ulcers are a type of skin ulcer characterized by a painful sore (usually of the lower leg), accompanied by disintegration of tissue which mostly affects the epidermis, often portions of the dermis, or even subcutaneous fat. Its management requires the generalized use of topical and systemic analgesia. However, these agents often cause severe systemic adverse effects. Some recent evidence suggests that topical instillation of sevoflurane on the vascular ulcer has a rapid, intense, and durable anesthetic and healing effect.

Peer-review

The study is detailed and well-made and the paper accurately written. The results reported in this study have been obtained by gas chromatography and NMR experiments, clearly and precisely described. This paper is worthy of publication.

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