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REVIEW ARTICLE

Mitochondrial Dysfunction in Stroke: Implications of Stem Cell Therapy

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Abstract

Stroke is a debilitating condition which is also the second leading cause of death and disability worldwide. Despite the benefits and promises shown by numerous neuroprotective agents in animal stroke models, their clinical translation has not been a complete success. Hence, search for treatment options have directed researchers towards utilising stem cells. Mitochondria has a major involvement in the pathophysiology of stroke and a number of other conditions. Stem cells have shown the ability to transfer mitochondria to the damaged cells and to help revive cell energetics in the recipient cell. The present review discusses how stem cells could be employed to protect neurons and mitochondria in stroke and also the various mechanisms involved in neuroprotection.

Keywords Stroke · Mitochondria · Reactive oxygen species · Neuroprotection · Tunnelling nanotubes · Extracellular vesicles · Cell fusion

Introduction

The powerhouse of the cell, mitochondria, are involved in regulating essential functions of the cell by generating the energy reserves, adenosine triphosphate (ATP). They are also involved in numerous other processes within the body such as energy metabolism, cellular survival, apoptotic cell death, free radical generation and maintaining intracellular calcium homeostasis [1, 2]. Around 90% of the cellular energy is supplied by the mitochondria via the electron transport chain (ETC) [3].

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Mitochondria are cytoplasmic, double-membrane organelles which generate energy in the form of ATP by two major steps via the oxidative metabolism of nutrients: (a) oxidation of dihydro-flavin adenine dinucleotide ($FADH_2$) or nicotinamide adenine dinucleotide (NADH) which are produced in the tricarboxylic acid cycle (TCA), glycolysis or during the beta oxidation of fatty acids and (b) ATP generation by oxidative phosphorylation (OXPHOS) [1]. The OXPHOS process is driven by an electrochemical gradient generated across the inner mitochondrial membrane (IMM). The mitochondrial matrix is surrounded by the IMM, wherein the ETC takes up the electrons generated in the TCA cycle for ATP synthesis. The IMM, which has enzymes that are involved in the process of ETC and ATP production, has a restricted permeability in contrast with the outer mitochondrial membrane (OMM) [4].

In normal aerobic respiration, about 2% of the total electrons leak out of the ETC via the complexes I and III. This leakage of electrons is responsible for the formation of free radicals within the mitochondria [3, 5, 6]. Although within optimal levels, these free radicals have physiological significance such as in inflammation [7]. However, uncontrolled production of free radicals can hamper the balance between free radical production and the body's antioxidant defence mechanism eventually leading to oxidative stress. Such a situation is observed in numerous disorders such as stroke,

myocardial infarction, mitochondrial dysfunction, atherosclerosis, Alzheimer's disease, Parkinson's disease, ageing etc. [8–13].

Stroke is devastating, and in incidences of ischaemic stroke where the occlusion is not reversed within the optimal time window, tissue infarction and cell loss occur in brain eventually leading to disability and death [14–17]. Risk in individuals increase with advancement of age, with women being more prone [18, 19]. Incidences of stroke nowadays are also being common in young adults, comprising 10–15% of all the stroke patients. Stroke in the younger population has a bigger impact as patients are left disabled during their productive years [20]. Systemic thrombolytics has been proven to be successful when used within the 3–4-h time window. Despite its benefit, it is difficult to re-establish the conditions that existed prior to stroke [21, 22]. Mechanical thrombectomy offers an alternative wherein clot retrieval devices are used. However, search for alternative forms of therapy is still necessitated [23, 24].

As mitochondria plays a pivotal role in the pathophysiology of stroke, this review manuscript focuses on its differential involvement in stroke. The various means by which one can target mitochondria has also been briefly discussed. A section has also been dedicated towards utilising stem cell therapy as a measure for preventing mitochondrial dysfunction in ischaemic stroke.

Pathophysiology

Ischaemic stroke is a condition that arises as a consequence of an occlusion in the cerebral arteries which ultimately leads to brain tissue infarction with loss of neuronal and glial cells [15, 25, 26]. The ischaemic region is characterised as the central core and the peripheral penumbra. The penumbra is usually defined as the region where the cerebral flow of blood is reduced to a level to cause hypoxia, capable of arresting physiological functions, but not reduced to a level to induce necrosis or irreversible loss of energy metabolism [27]. Core is defined as the central portion of the ischaemic region where there is irreversible tissue damage and complete loss of energy metabolism [28]. Restoration of the cerebral circulation, although effective in re-establishing oxygenation, often leads to reperfusion injury to the affected area [29]. A number of cellular and molecular mechanisms are believed to be involved in reperfusion injury that involve adaptive and innate immune system and also the platelets, coagulation factors and the complement system [30, 31]. Once these systems are activated, cell death can occur via apoptosis or necrosis, which further activate the inflammatory system to cause a more extensive reperfusion injury [32, 33]. Mitochondria plays a pivotal role in exacerbating reperfusion injury by producing free radicals in excess [34, 35].

Mitochondrial pathophysiology has become a focal area of research in recent years. Furthermore, as mentioned earlier, mitochondria serve as the lifeline of most cells due to their indispensable role as source of energy production. Mitochondria have a circular genome (mitochondrial DNA (mtDNA)) which is 16.6 kb in mammals, encoding 13 subunits and have 2 ribosomal RNA (rRNA) and 22 transfer RNA (tRNA) [36]. These subunits form an essential component of the ETC. There are multiple copies of the mitochondrial genome, organised as mtDNA-protein complexes called nucleoids [37]. Mutations within the mtDNA can lead to pathologies such as neurodegenerative disorders, cancer etc. [38]. The ETC comprises five complexes on the IMM that constitutes a large structural and functional unit of the mitochondria and is responsible for running a series of redox reactions that drives the phosphorylation of adenosine diphosphate (ADP) to ATP [39]. NADH and $FADH_2$ are the coenzymes that donate electrons from the TCA cycle to the complex I (NADH ubiquinone reductase) or complex II (succinate dehydrogenase) of the ETC. The electrons are then transferred to complex III (ubiquinol-cytochrome *c* reductase), followed by complex IV (cytochrome *c* oxidase) and finally through complex V (ubiquinol-cytochrome *c* reductase) [40]. This electron transfer across the ETC is coupled with proton transfer across the IMM from complexes I, III, IV and V (Fig. 1). This establishes a proton gradient across the IMM which is utilised for ATP synthesis [1]. Therefore, interruption in this energy supply results in detrimental implications for the cell and threatens its survival. In the ETC, oxygen is continuously getting metabolised and generates reactive oxygen species (ROS). Often, there is incomplete reduction of oxygen in the mitochondria leading to production of ROS, such as superoxide anion ($\cdot O_2^-$), hydroxyl radicals (·OH) and other reactive species, such as reactive nitrogen species (RNS) and nitric oxide (NO) [6, 26].

One of the major driving forces of mitochondrial pathology is the damage incurred by oxidative stress and the involved ROS [41]. Studies have reported mitochondria serving as sources of ROS, as much as 5%, due to their production of the superoxide anion and hydroxyl radicals [42]. (·OH) can attack and breakdown the integrity of the mitochondrial membrane along with its essential proteins and nucleic acids [43]. The internal breakdown of the ETC by these radicals entails consequential genomic instability and hinders expression of proteins vital for oxidative phosphorylation, sending the respiratory machinery into a vicious cycle of exaggerated ROS production that threatens to cause mitochondrial destruction and other cellular components once they leak into the cytosol [44]. The events eventually signals for the apoptotic cell death. The complexes of the ETC are reported to play essential roles in pathological conditions. Complex I has been reported to be associated with the majority of the mitochondrial pathologies as well as in neurodegenerative disorders [45]. Its proclivity

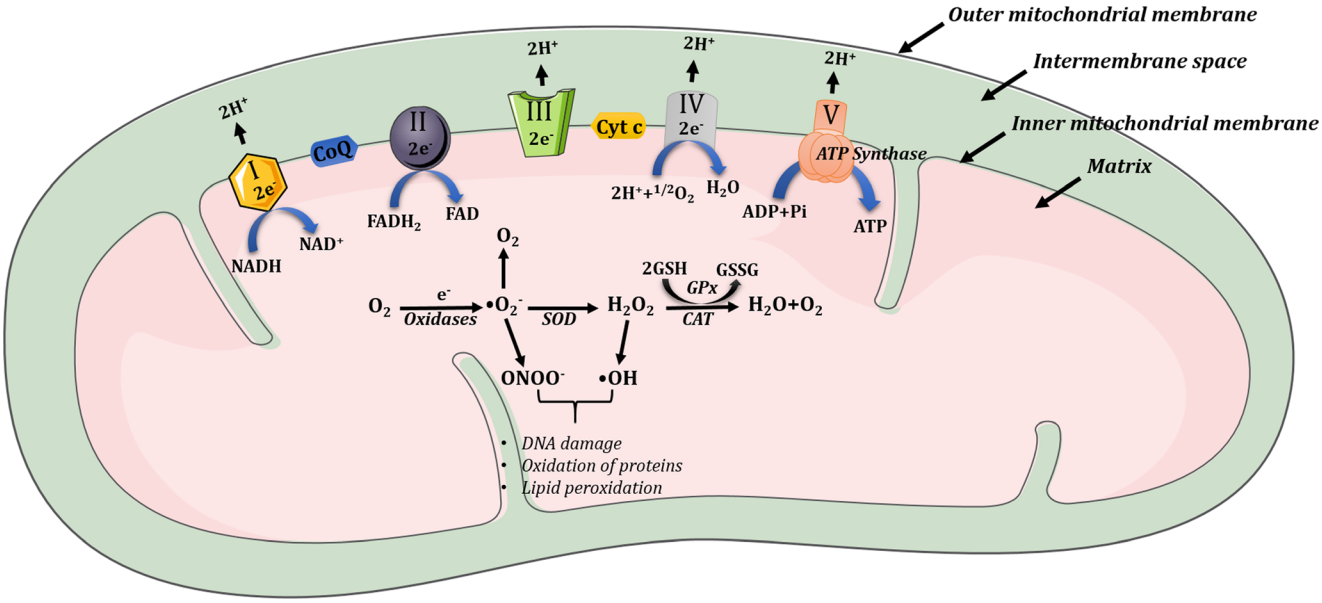

Fig. 1 Electron transport chain (ETC). ETC of the mitochondria is composed of five complexes situated in the IMM. The co-enzymes of the TCA cycle, NADH and $FADH_2$, are accepted within the ETC and transferred to complex I (NADH ubiquinone reductase) or II (succinate dehydrogenase). From these, the electrons are then transferred to complex III (ubiquinol-cytochrome *c* reductase), IV (cytochrome *c* oxidase), and ultimately through complex V (ubiquinol-cytochrome *c* reductase) to oxygen. The electron transport is linked with proton transfer by complexes I, III, IV and V across the IMM, establishing a proton gradient which is utilised for ATP synthesis. During the process of electron transfer across the ETC, there is generation of $\cdot O_2^-$, which gets converted by SOD to H_2O_2. H_2O_2 in the presence of enzymes CAT and GPx is converted to water. Excessive free radical generation can cause DNA damage, protein oxidation and lipid peroxidation. IMM, inner mitochondrial membrane; TCA, tricarboxylic acid cycle; NADH, nicotinamide adenine dinucleotide; $FADH_2$, dihydro flavin adenine dinucleotide; ATP, adenosine triphosphate; $\cdot O_2^-$, superoxide anion; SOD, superoxide dismutase; H_2O_2, hydrogen peroxide; CAT, catalase; GPx, glutathione peroxidase

towards generating a larger amount of ROS stems from its structural and functional configuration; the radicals arise through two different mechanisms. Housing a flavin mononucleotide (FMN) factor that serves as an entry point for electrons shuttled by NADH to the rest of the respiratory chain, complex I is vulnerable to breakdown and causing a major backup of electrons [6]. Since the NADH/NAD+ pool dictates the rate of redox-dependent transport of electrons down the chain, it is vital that the FMN factor quickly transfer the electrons to the seven iron-sulphur (FeS) sites and onto the waiting ubiquinone carrier, thereby maintaining an optimal and safe equilibrium between NADH and NAD+. However, disruption to complex I, via rotenone inhibition (and other damage, mutation, cytochrome *c* loss or accumulation of NADH due to a lack of ATP demand), can throw a cog into the chain of electron movement, causing a massive backup of electrons near the FMN site and raising the NADH/NAD+ level. The released electrons are involved in the reduction of oxygen (O_2) to the superoxide anion (O_2^-) [46]. Another mechanism that makes complex I highly prone to generating ROS occurs in a similar manner, but in this, a ubiquinone binding site is involved in which a high proton motive force is coupled to reverse electron transport [47]. Though exact site of O_2^- generation is still unclear, previous studies suggest that semiquinolates formed during proton pumping may directly react with O_2 to form O_2^- [47]. Complex II has been shown to detect apoptotic signalling due to the acidification induced by proapoptotic compounds (Fas ligand (FasL)) by the dissociation of the succinate dehydrogenase complex flavoprotein subunit A/B (SDHA/B) subunits in complex II, leading to an uncoupled complex II, ROS production and cell death [48].

When ROS accumulates and compromises mitochondrial function and threatens the rest of the cell, a cascade of signals triggers the mitochondria to elicit apoptosis. A few studies have firmly established that the apoptotic cell death is mediated by two pathways viz. intrinsic and extrinsic [49]. In brief, the intrinsic pathway consists of pro-apoptotic factors bound to the outer mitochondrial membrane (OMM) and catalyse the formation of a permeable transition pore (mPTP) which releases mitochondrial intermembrane space proteins including second *mitochondria*-derived activator of caspases (Smac), *apoptosis*-inducing factor (AIF) and cytochrome *c* [50] into the cytosol. Cytochrome *c* interacts with apaf-1 to form an apoptasome and converts procaspase-9 to the active caspase-9, which further triggers the execution phase that involves caspase 3 activation. Smac is also involved in the activation of caspases, it binds to and neutralises the X chromosome-linked inhibitor-of-apoptosis protein. This inhibits procaspase activation and the activities of the activated caspases [51]. Following ischemia, AIF translocates to the nucleus

from the mitochondria and induces apoptosis [52–54]. The activation of caspase 3 sends the cell into a programmed self-destruction mode where various enzymes (endonucleases and proteases) degrade chromosomal DNA and spur nuclear fragmentation; the process is an organised breakdown of cellular components through the expression of ligands for phagocytic receptors for phagocytosis [49]. Meanwhile, the extrinsic pathway is initiated by FasL or tumour necrosis factor alpha (TNF-α) at their respective receptors to recruit adapter proteins to assemble death domains near the cytosolic receptor terminals and associate with pro-caspase 8 to induce a death-induced signalling complex (DISC), which catalyses and activates the pro-caspase 8 to caspase 8 and triggers the execution phase of apoptosis, which falls in line with that of the intrinsic pathway [49]. Perforin-granzyme-dependent cell death is induced by attacking T cells, but the execution phase falls in line with that of the extrinsic and intrinsic pathways [55]. Necrosis and aponecrosis are the other death pathways that are energy independent and are toxic processes in which the swelling cell bursts in a disorganised manner and is detrimental to nearby cells [49].

The pro-apoptotic factors, such as B cell lymphoma 2 (Bcl-2)-associated X protein (BAX) and Bcl-2-associated death promoter (BAD), are kept in check by phosphokinases, such as protein kinase B (Akt), that render them inactive and maintain the survival signalling of a cell active [56]. However, during excitotoxicity imparted by large calcium influx, a calcium/calmodulin phosphatase, calcineurin (CaN) (Fig. 2), becomes activated and dephosphorylates the pro-apoptotic factors and activates them [57]. Activated BAD then translocates to the OMM and forms a complex with anti-apoptotic factors, Bcl-2 and B cell lymphoma-extra large (Bcl-xL), and renders them inactive, therefore eliciting signals to BAX to move to the OMM and form the mPTP and thereby releasing cytochrome *c* and facilitating the formation of the apoptasome. The rest of the pathway follows the execution phase and leads to cell death [58]. CaN can also trigger apoptosis through the dephosphorylation of dynamin-related protein 1 (Drp1), causing it to set off mitochondrial fission by attaching to the OMM and forming puncta that sever and cleave the mitochondria [59]. Although, mitochondrial fission serves as a protective mechanism in a normal cell, it can also indicate a pathological condition; spherical mitochondrial debris void of cytochrome *c* indicate apoptotic events triggered by an underlying pathology [60].

Targeting Mitochondria

Previous studies have established the involvement of mitochondrial dysfunction in stroke, neurodegenerative diseases, ageing and other metabolic disorders. Hence, targeting this powerhouse of the cell appears to be a potential therapeutic approach for treating mitochondria-associated pathology. A number of such strategies have been discussed in the following paragraphs which can be employed to reduce ROS that causes the oxidative stress leading to mitochondrial dysfunction.

Lifestyle Changes Studies have shown that in type 2 diabetes mellitus, physical activity propound various benefits through mitochondrial biogenesis and enhanced insulin sensitivity in skeletal muscles [1]. It was observed that following exercise, 5′ adenosine monophosphate-activated protein kinase (AMPK) gets stimulated, which phosphorylates threonine and serine residues and activates peroxisome proliferator-activated receptor gamma coactivator 1 (PGC 1) [61]. Activation of this signalling pathway leads to a significant increase in mitochondrial biogenesis, its content, improves muscle mitochondrial respiration, increase oxidative enzymes and mitochondrial density in type 2 diabetes patients [1, 61]. Calorie restriction (CR) is also found to operate a protective and preventive intervention that acts against metabolic disorders and prolongs the lifespan of individuals [62]. The mechanism behind the protective effects of calorie restriction is still not clear, but several studies have described that by restricting calories, one can reduce the elevated levels of reactive oxygen species (ROS) and oxidative damage in the direction for the improvement of deregulated mitochondrial functions in humans [63–65]. CR is also involved in mitochondrial biogenesis as it enhances the levels of NAD^+ in tissues, which via activation of SIRT1 deacetylates PGC1α [62].

Antioxidant Therapies Another potential therapy for mitochondria-associated diseases include antioxidant therapies. Antioxidants targeted towards mitochondria in metabolic disorders have shown beneficial effects in both in vivo and in vitro studies. Agents with antioxidant properties such as coenzyme Q, N-acetylcysteine, vitamins C and E etc. are administered to patients with metabolic disorders that helps to regulate the levels of ROS within the system [3]. The need for mitochondria-targeted therapy has led to designing of molecules that specifically accumulate within the mitochondria [66]. One such molecule is MitoQ, a derivative of ubiquinone which is conjugated with a lipophilic cation, TPP [67, 68]. This lipophilicity allows the molecule to enter the cell and accumulate within the mitochondria via electrochemical gradient [3]. Ubiquinone reduces the lipid peroxyl production and prevents lipid peroxidation [67, 68]. Due to its beneficial effects in vitro and in vivo, MitoQ has been tested for liver damage and Parkinson's disease in clinical trials [69, 70]. Tiron, an iron chelator and an antioxidant, permeabilize the mitochondrial membrane and accumulate within the organelle [71, 72]. Protective effects of tiron in human dermal fibroblasts against photoaging has been demonstrated by Fang et

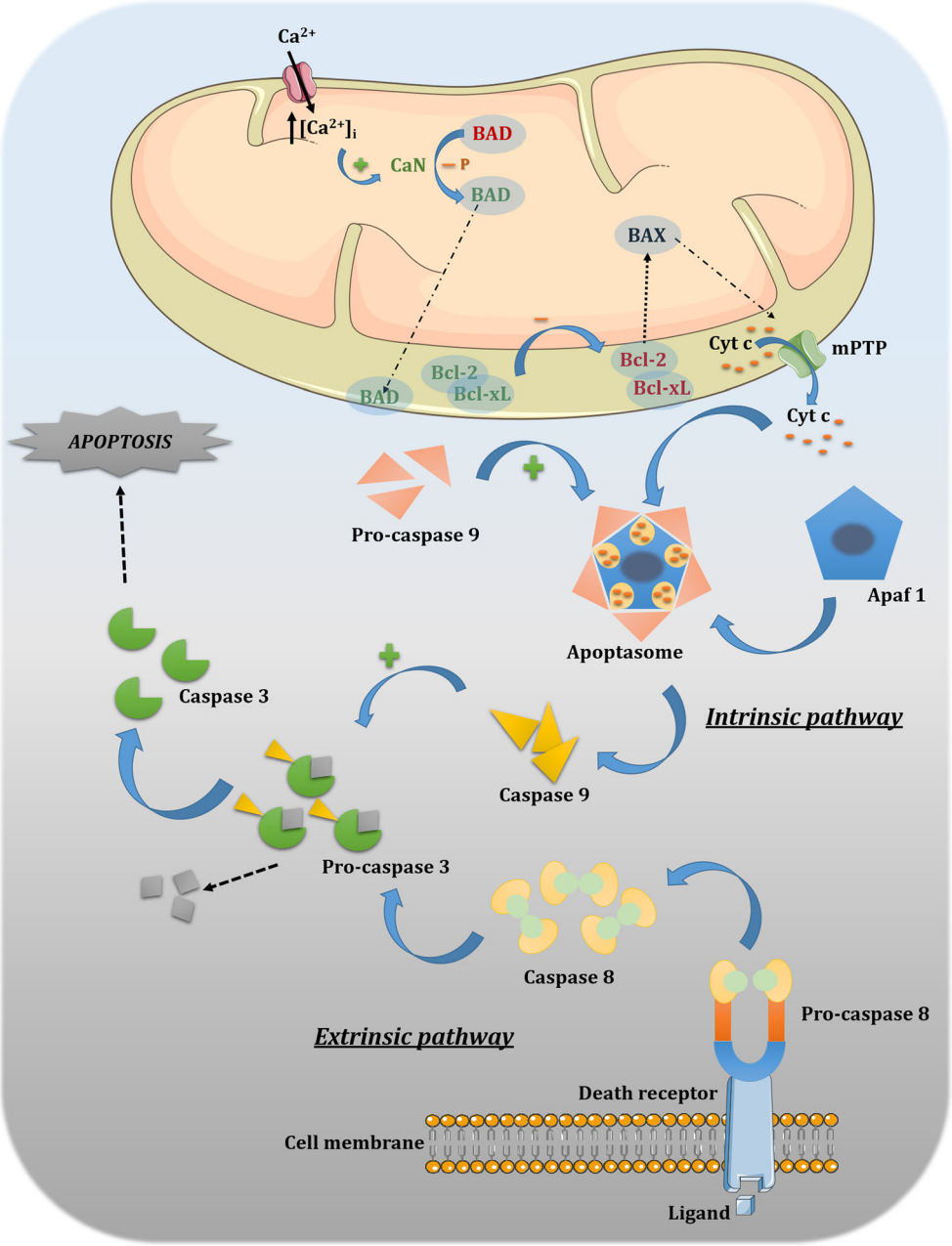

Fig. 2 Role of mitochondria in the pathophysiology of stroke. Following influx of calcium in the mitochondria, there is activation of CaN which dephosphorylates BAD, which further uncouples Bcl-2 and Bcl-xL and activates BAX. This leads to formation of mitochondrial permeability transition pore (mPTP) and the release of cytochrome *c* (cyt *c*). Following the release of cyt *c*, the cell follows the intrinsic cell death pathway. In the extrinsic cell death pathway, following activation of the death receptor by its ligand, pro-caspase 8 is converted to caspase 8, which then integrates with the intrinsic pathway at the level of pro-caspase 3. CaN, calcineurin; mPTP, mitochondrial permeability transition pore; Ca, calcium; BAD, Bcl-2-associated death promoter; Bcl-2, B cell lymphoma 2; Bcl-xL, B cell lymphoma-extra large; BAX, Bcl-2-associated X protein; cyt *c*, cytochrome *c*

al. [72]. Tiron works by inhibiting the production of oxygen radicals induced by ultraviolet-B (UV-B) [3, 72]. Study by Mailloux suggested that mitochondria-targeted glutathione (GSH) analogues might participate in various cardiovascular diseases by allowing the direct restoration of GSH levels and protect mitochondrial redox buffering and its signalling capacity. Thereby, mitochondria-penetrating antioxidant proved to be beneficial in the treatment of cardiovascular diseases. Mitochondria-targeted antioxidants such as vitamin E (MitoVit E) or ubiquinone (MitoQ) are helpful in reduction of ROS against mitochondrial dysfunction [1, 73]. Mao et al. found that MitoVit E reduced hepatic oxidative stress and hampered fat deposition in mice [74].

It was also found that untargeted antioxidants such as vitamin E have 350-fold low potency when compared with mitochondria-targeted antioxidant MitoVit E. Mitochondria-targeted antioxidants were proved to be of therapeutic potential in the treatment of Friedreich Ataxia, Huntington's disease, Alzheimer's disease by preventing amyloid beta toxicity, Parkinson's disease, oxidative stress associated with haemorrhagic shock and reperfusion and other disorders involving mitochondrial oxidative damage [73, 75–79].

NXY-059, a free radical trapping agent, showed immense potential as a neuroprotective agent in animal models of acute ischaemic stroke (AIS). It was able to reduce the volume of cerebral infarction and also improve functional recovery.

However, the agent was found to be ineffective in clinical trials for treating AIS. The study suggested that animal models are not relevant to patients, and there is a need to revaluate the strategies employed for the development of neuroprotective drugs [66, 80]. Another agent, Stilbazulenyl nitrone (STAZN), a potent antioxidant, when administered intravenously in rats with middle cerebral artery occlusion (MCAo), demonstrated improved neurological outcome and reduction in infarct volume. This agent was able to confer neuroprotection even in low doses (0.7 mg/kg). STAZN, in contrast with NXY-059, is more lipophilic and is expected to have a higher penetration through the blood–brain barrier (BBB) [81]. At the same time, STAZN being more potent, effectively inhibits lipid peroxidation [82]. Further studies are still warranted to look into its neuroprotective potential, for which proper design of pre-clinical and clinical trials are required [83] (Table 1).

Pharmacological Strategies Once oxidative stress takes place, structural and functional changes are seen in mitochondria of the cells. The damaged mitochondria activate a number of signalling pathways which can produce ROS in an uncontrolled manner leading to various diseases and cellular death [84, 85]. Targeting these pathways by drugs presents one such approach by which mitochondrial function can be protected by limiting the ROS production. Sirtuin (silent mating type information regulation 2 homologue) 1 (SIRT1), a NAD-dependent deacetylase enzyme, is known to enhance mitochondrial function and reduce oxidative stress [86]. They act as redox state and cellular energy sensor and are regulated by the cellular metabolic conditions [87]. SIRT1 is also known to regulate metabolism of lipid and glucose by insulin signalling in the skeletal muscles, adipose tissues and liver [88–92]. An important activator of SIRT1, resveratrol, has been shown to possess antioxidant properties [1]. *SIRT1* gene activation protects cells from inflammation and oxidative stress via PGC1α, a transcription co-activator of peroxisome proliferator-activated receptors (PPARs) and promotes mitochondrial biogenesis and glucose uptake [1, 87, 93]. Resveratrol pretreatment has shown to protect rat brain from damage following ischemia via the SIRT1 uncoupling protein 2 pathway (SIRT1-UCP2) [94]. Along with ROS, mitochondrial fission has also been implicated in various disorders and can be targeted for treating patients [95]. Dynasore, mitochondrial division inhibitor 1 (Mdivi1) and mitochondrial fission peptide inhibitor (P110) are the three mitochondrial fission inhibitors which play a protective role against oxidative stress [96–98].

Purines also have the ability to act as neuroprotective agents. Following hypoxia, there is a rapid efflux of ATP to the extracellular space. This extracellular accumulation of adenosine has shown to reduce ischaemic damage [99, 100]. Cellular energy levels can be maintained through excitation of exogenous purinergic receptors [101]. Neuronal hyper-excitability, a hallmark of ischemia, is thought to underlie the associated neurodegeneration through enhanced glutamate release and disruption in calcium homeostasis that can be alleviated by purinergic receptor agonist [102]. A potent, selective purinergic agonists MRS2365 and 2-methylthioadenosine diphosphate trisodium salt (2meSADP) increase astrocyte mitochondrial metabolism via purinergic (P2Y1) receptor activation which can provide neuroprotection following stroke [103–106]. Studies have been conducted wherein 2meSADP have been used in Rose Bengal photothrombosis model of stroke [107–109]. This drug was able to markedly reduce the infarct size in mice [110]. It was also observed that oedema formation was reduced and neuronal survival increased following 2meSADP administration [110]. Mitochondrial membrane potential levels were also found to be normalised. All morphological changes associated with neuronal death were reversed [109]. In a rat MCAo model of ischemia/reperfusion, rats administered with 2meSADP during reperfusion had shown reduction in infarct volume as confirmed using 2,3,5-triphenyltetrazolium chloride (TTC) staining, 48 h following stroke [101] (Table 1).

Methylene blue, a *Food and Drug Administration* (FDA)-approved agent, has been employed for neurodegenerative disorders such as Parkinson's and Alzheimer's diseases [111]. Methylene blue acts as an alternate electron carrier by transferring electrons between NADH and cytochrome *c*, thereby creating a mechanism of bypassing the complexes I and III. In this manner, methylene blue reduces the electron leakage from the ETC and assists in continuous ATP production [101]. Methylene blue keeps under check the amount of ROS produced, thereby limiting neuronal damage by oxidative stress [101]. This agent has shown in vitro to improve mitochondrial function, as demonstrated by Wen et al. [112]. They also established that the compound is protective in rat intraluminal filament MCAo model. Five hundred microgrammes per kilogramme dose of the agent was able to enhance the activity of complexes I, II and IV [112]. In in vivo studies with rats, methylene blue was able to preserve cerebral blood flow and glucose uptake as compared with normoxic rats when exposed to hypoxic conditions [113]. Methylene blue also reduced behavioural deficits as well as the infarct size in a rat 60 min transient cerebral ischemia model, as seen by Huang et al., using non-invasive magnetic resonance imaging [114]. Thus, these results support exploring methylene blue as a stroke intervention.

During hypoxia, there is an imbalance between the endogenous antioxidants of the body and ROS which leads to injury and cell death. Superoxide dismutases (SOD) are a group of enzymes in the mitochondria, catalysing the conversion of superoxide anion, a radical which is responsible for the damage following ischemia, to oxygen and hydrogen peroxide [101, 115]. These enzymes are known to have neuroprotective

Table 1 Table showing the various strategies available for targeting mitochondria

Sl. No.	Agent	Mechanism of action	Reference
Antioxidant therapies			
1.	Coenzyme Q	Regulate ROS levels	[3]
2.	N-Acetylcysteine	Regulate ROS levels	[3]
3.	Vitamin C	Regulate ROS levels	[3]
4.	Vitamin E	Regulate ROS levels	[3]
5.	MitoQ	Reduce lipid peroxyl production and prevents lipid peroxidation	[67, 68]
6.	Tiron	Inhibits the production of oxygen radicals	[71, 72]
7.	Glutathione analogues	Restore glutathione levels	[73, 74]
8.	MitoVitE	Reduce ROS	[74]
9.	NXY-059	Free radical trapping agent	[66, 80]
10.	STAZN	Inhibits lipid peroxidation	[82]
Pharmacological strategies			
1.	Resveratrol	Activates SIRT1 which promotes mitochondrial biogenesis	[93]
2.	Dynasore	Mitochondrial fission inhibitor	[96–98]
3.	Mdivi1	Mitochondrial fission inhibitor	[96–98]
4.	P110	Mitochondrial fission inhibitor	[96–98]
5.	MRS2365	Purinergic agonist, increase astrocyte mitochondrial metabolism	[104]
6.	2meSADP	Purinergic agonist, increase astrocyte mitochondrial metabolism, normalise mitochondrial membrane potential	[109]
7.	Methylene blue	Reduces electron leakage from ETC, keeps check on the amount of ROS produced.	[102]
8.	Mn(II) pentaazomacrocyclic complexes (M40403, mitoSOD)	SOD mimetic	[101, 124]
9.	Mn(III) porphyrins (MnTm4PyP)	SOD mimetic	[122]
10.	Mn(III) salen (EUK8, EUK13 and EUK134)	SOD mimetic, reduce nitrosative stress and oxidative stress	[120]
11.	Cu-Zn SOD	Inhibits apoptotic neuronal cell death	[125]
12.	Methazolamide	Inhibits ROS production, inhibits neuronal apoptosis/caspase-3 activation	[126]

effect and counteract the injuries mediated by ROS following cerebral ischemia [116]. Deficiencies of these enzymes are linked to worsening of cerebral infarcts. In transgenic mice, overexpression of SOD 1 and 2 enzymes proved to be beneficial and protective [117, 118]. However, low oral bioavailability, short half-life, instability and high molecular weight make its use as a therapeutic agent limited. To overcome this drawback, SOD mimetics are currently available which are under scrutiny. These mimetics have low molecular weight, non-peptide, easily diffuse and permeate through the cell, non-immunogenic, have similar efficacy as the native SOD and are not inactivated by peroxynitrate [119]. Mn(II) pentaazomacrocyclic, Mn(III) porphyrins and Mn(III) salen complexes are the three main classes of SOD mimetics, where the metal complexes within the molecule mimics the endogenous enzyme's active site and redox potential of the magnesium ion plays an important role in their relative activities [120, 121]. Manganese(III) tetrakis(1-methyl-4-pyridyl)porphyrin (MnTm4PyP), a porphyrin, has been demonstrated both in vitro and in vivo to be neuroprotective. In a mouse MCAo model, MnTm4PyP showed a dose-dependent decrease in cytochrome *c*, superoxide radical and cleaved caspase-3 [122]. SOD2 mimetics, as compared with control, maintained intracellular cytosolic calcium levels and reduced superoxide levels in in vitro analysis of primary cortical neuron [123]. M40403, a Mn(II) pentaazomacrocyclic mimetic, is highly selective for superoxide radical and does not react with other ROS example peroxynitrite, hypochlorous acid and hydrogen peroxide. In vivo studies have shown that this compound is having a neuroprotective effect and is highly stable. However, its uptake within the energised mitochondria is negligible [124]. Potential-driven uptake of M40403 into the mitochondria was improved by linking a lipophilic cation triphenylphosphonium (TPP), the compound was named MitoSOD. The defence potential of endogenous SOD was surpassed by mitoSOD against ROS [101, 124]. EUK8, EUK13 and EUK134, SOD mimetics of the Mn(III) salen class have also shown ability to reduce nitrosative stress and oxidative stress [120]. Further studies are warranted to assess the efficacy of these SOD mimetics. Copper–zinc (Cu–Zn) SOD has been shown to protect brain during an ischaemic episode and its reduced levels is known to be involved in

formation of oedema and exacerbate neuronal cellular injury. Cu–Zn SOD is involved in the modulation of neuronal viability by inhibiting apoptotic neuronal cell death following some form of cellular damage [125] (Table 1).

In a mouse model of subarachnoid haemorrhage, methazolamide, a neuroprotective agent against ischaemic stroke has shown to significantly improve neurological behaviour by inhibiting neuronal apoptosis/caspase-3 activation. At the same time, methazolamide administration reduces oxidative stress through effectively inhibiting ROS production in primary cortical neuron induced by blood exposure or haemoglobin insult [54, 126].

Stem Cells in Mitochondrial Dysfunction

In the previous section, we have discussed the different approaches available for targeting mitochondria. Apart from antioxidant and pharmacological strategies, stem cell therapy has also come up as an alternative. Several pre-clinical and clinical studies have shown their support towards utilising stem cells as therapeutic agents in disorders where mitochondrial dysfunction plays an important role [127–130]. Hayakawa et al. showed that in mice, transient focal ischemia could induce the entry of mitochondria from astrocytes into the adjacent neurons which amplified cellular survival signals [131]. This study proves that mitochondrial transfer is possible from astrocytes to neurons after stroke and in a similar manner stem cells can also be beneficial. Stem cells can migrate towards the injury site following transplantation, where they can give rise to new functional neurons and form connections with the host cells [18]. Intercellular mitochondrial transfer for replacement of damaged mitochondria has emerged as an additional mechanism [38]. This mechanism was first observed with human mesenchymal stem cells (MSCs), wherein healthy mitochondria could be transferred to cells deficient in mitochondria for rescuing their aerobic respiration [132]. Since this observation, the in vitro transfer of mitochondria to different cell lines has been extensively studied, and it has been suggested that such transfer can restore mitochondrial functioning, rescue stressed cells and at the same time reprogram differentiated cells [132–138].

Exchange of organelles demonstrate a unique form of cellular communication which permits the transfer of small ions, molecules, signals as well as intracellular structures like mitochondria, endosomal vesicles, lysosomes etc. both uni- as well as bi-directionally [139]. Such transfers allow the incorporation of the mitochondria into the host's mitochondrial network and make changes within its bioenergetics and other functional properties, both in vitro and in vivo. The exact mechanisms as to how these mitochondria are transported to the host cells are yet to be completely explained. Transfer of mitochondrial genes also accompanies the intercellular mitochondrial transfer which has important implication in the pathophysiology of mitochondrial dysfunction [135]. Spees et al., as mentioned, were the first to provide evidence of mitochondrial transfer for rescuing mitochondrial respiration between human stem cells and mitochondria-depleted cells [132]. Such transfer involved active processes of actin-based tubes, tunnelling nanotube (TNTs) formation or the vesicular transfer of mitochondrial fragments or mitochondrial DNA (mtDNA) rather than involving passive uptake of mitochondrial fragments (Fig. 3a) [38]. Majority of the studies have utilised stem cells as mitochondria donors [140]. Studies carried out by Liu et al. indicated mitochondrial transfer from MSCs to human umbilical vein endothelial cells (HUVEC) initially subjected to ischaemic-reperfusion injury in vitro [141]. This transfer was able to restore the aerobic respiration in these endothelial cells, when compared with cells which were cultured alone or those MSCs harbouring dysfunctional mitochondria. They also found that the damaged cells expressed phosphotidylserine, triggering MSCs towards formation of TNTs and thereby acting as a guide to the injured cells [141]. Similarly, MSCs improved survival and repaired cellular damage by the similar mechanism in cardiomyocytes which were previously subjected to glucose-oxygen deprivation for inducing ischemia followed by reperfusion [142]. Cigarette smoke-induced lung damage was attenuated following mitochondrial transfer from MSCs to lung epithelium [138]. It has been demonstrated that the mitochondrial transfer efficiency is higher in iPSC-MSC than that of BM-MSC in rescuing mitochondria damaged by cigarette smoke [138]. Plotnikov et al. showed that mitochondrial transfer to lung epithelium and endothelium cells from MSCs is an important mechanism by which these stem cells demonstrate their protective effect in animal model of lung diseases [143]. Yorgov et al. showed that damaged mitochondria were engulfed by the MSCs and degraded, leading to activation of haeme-oxigenase-1 (HO-1) (a cytoprotective enzyme) and stimulate mitochondrial biogenesis, thereby increasing the capacity of MSCs to donate mitochondria towards the injured cells for overcoming oxidative stress [144].

The above-mentioned observations have led to the proposal that cellular stress is an important signal required for organelle transfer, and these transfers were not detected under conditions where the mitochondrial function was partially affected [38]. In in vivo experiments also, mitochondrial transfer has contributed to tissue repair, mainly by improving function and enhancing cellular bioenergetics. In a study by Islam et al., stem cells derived from bone marrow when infused into the trachea of mice, treated with lipopolysaccharide (LPS), attached to the epithelial cells of the alveoli by means of connexions [133]. Connexins following oligomerization forms GAP junctions that connect the two cells allowing transfer of small cellular components. Connexin 43 specifically regulates the formation of nanotubes and vesicles which help in mitochondrial transfer between the alveolar cells and stem

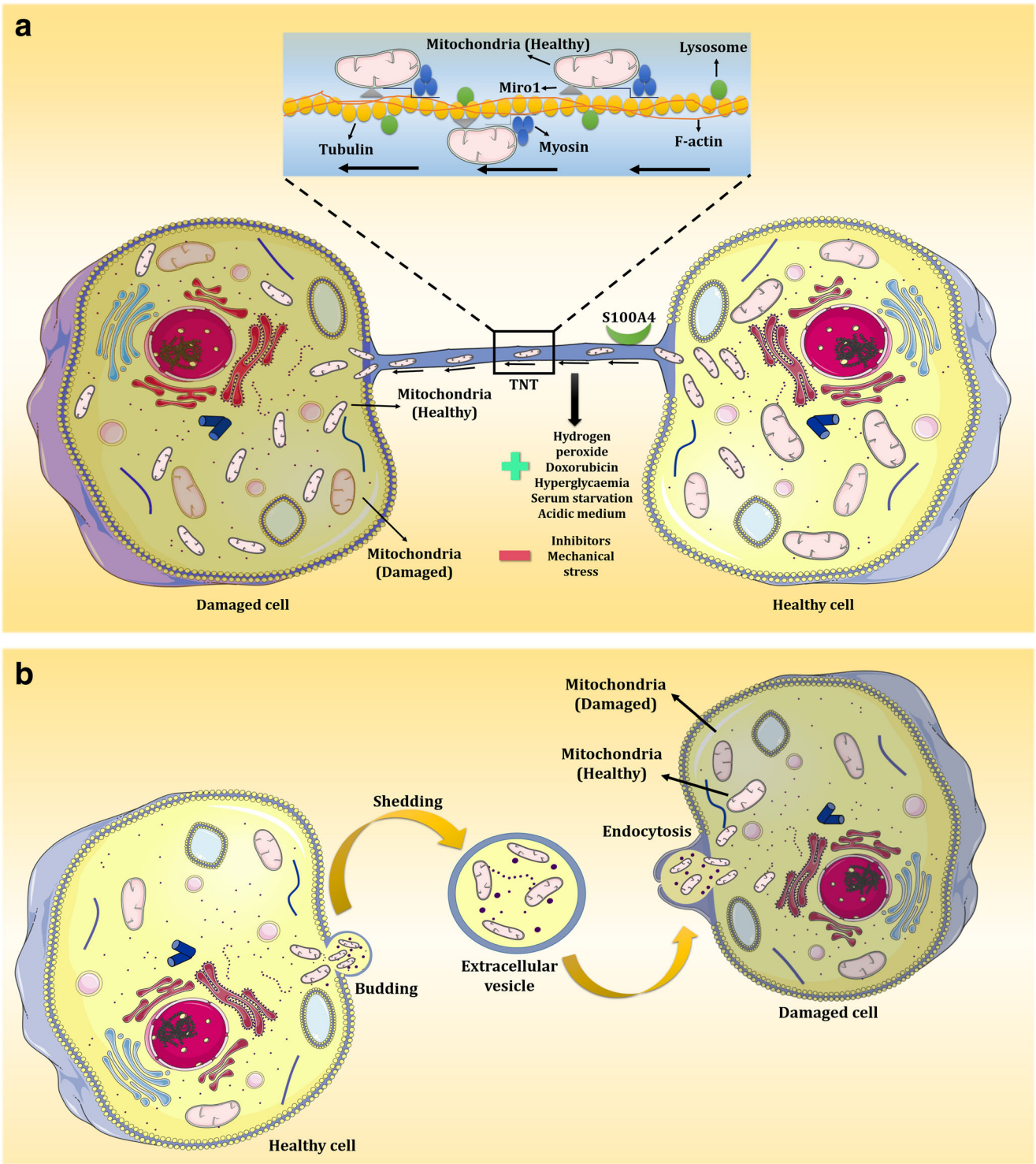

Fig. 3 Mechanism of mitochondrial transfer from stem cells. **a** Transfer of mitochondria via TNTs. TNTs facilitate the transfer of mitochondria from one cell to another. Hydrogen peroxide, doxorubicin, hyperglycaemia, serum starvation and acidic media stimulate the formation of TNTs, while TNT inhibitor and mechanical stress hamper the efficiency of mitochondrial transfer. S100A4 is an important protein, which along with its receptor, guides the TNT formation. TNT, tunnelling nanotubes; S100A4, S100 calcium-binding protein A4. **b** Extracellular vesicle-mediated mitochondrial transfer. Complete mitochondrial particles transfer mediates mitochondrial function rescue. **c** Mitochondrial transfer by cell fusion. Cellular fusion is triggered following stress. Transfer of mitochondria takes place once fusion between cells is established

cells. Following mitochondria transfer, there is increase in the ATP levels of the alveolar cells and an increased production of pulmonary surfactant [133]. A molecular mechanism for the mitochondrial transfer was explored in vitro and in mice

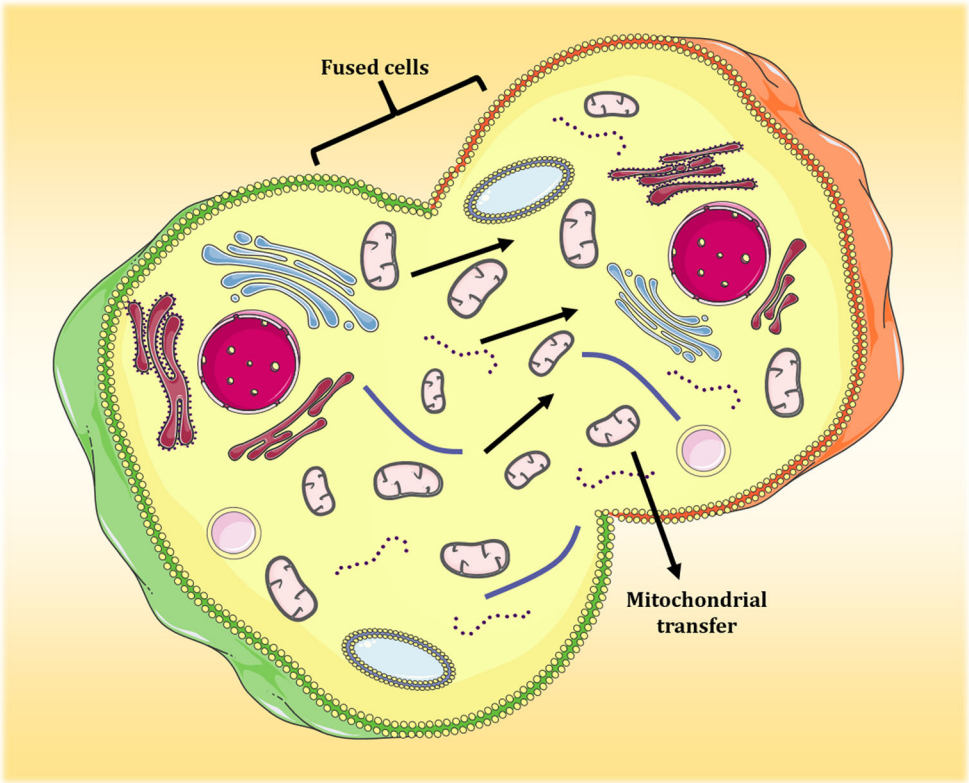

Fig. 3 (continued)

model of asthma, where it was found that the efficiency of intercellular mitochondria movement is regulated by Miro1, a Rho GTPase protein which connects mitochondria to cytoskeletal motor proteins. Ahmed et al., in this study found that MSCs which overexpressed Miro1 showed an increased mitochondrial transfer via TNTs towards the epithelial cells under stress. This was able to reduce inflammatory cell infiltration, cellular apoptosis, collagen deposition and hypersecretion of mucus in lungs [134]. Mitochondria transfer from stem cells to cancer cells has also been established. Transfer of mitochondria from bone marrow MSCs towards acute myelogenous leukaemia (AML) cells enhances survival and promotes chemoresistance in vitro to doxorubicin [145]. They also demonstrated that AML cells were able to receive up to 16 mitochondria, which represents 14% of their total mitochondrial mass, which is about 124 mitochondria per AML cell. The organelle transfer was also associated with a mean increase of 50% in ATP production by mitochondria along with 4.5-fold increase in ATP content [145]. However, a few important questions arises regarding the transfer of functional mitochondria, first, the degree of cellular impairment or damage that is a prerequisite for establishing a transfer between two cells and second, the mechanism by the which these cells sense the stress of the neighbouring cells to initiate mitochondrial transfer to restore cellular functionality, rather than moving the damaged cell towards apoptosis for its removal [38]. Further studies and experiments are required to find answers for these questions.

The mechanisms by which mitochondria are acquired by cells having dysfunctional mitochondria is still unclear. The signalling and molecular processes as well as its regulation remain elusive. Cells possess inherent mechanism by the virtue of which they can 'sense' the damage signals arising from cells under stress and they can initiate organelle exchange in response to these signals. TNTs are thought to be the major structures that mediate mitochondrial transfer inter-cellularly [38]. These TNTs have been demonstrated both in vitro and in vivo and facilitate the transfer of small cellular components like vesicles, membrane components and organelles. The emergence of a filapodium like membranous protrusion initiates the formation of a TNT. This protrusion is retracted once it reaches the recipient cell, leaving behind an ultrafine structure [146]. Studies with chemical inhibitors of TNT formation and mechanical stress have shown that TNTs are necessary for mitochondrial exchange and its inhibition reduces the efficiency of mitochondrial transfer. This exchange may be both unidirectional or bidirectional [146–148]. The proteinS100 calcium-binding protein A4 (S100A4), along with its receptor, is said to guide the TNT direction growth [149]. Agents or factors which enhance TNT formation include hydrogen peroxide, doxorubicin, mitochondrial damage, serum starvation, hyperglycaemia and acidic medium while stress can inhibit TNT formation [150].

The second mechanism of mitochondrial transfer is by means of extracellular vesicles (EVs) (Fig. 3b), which act as biomarkers for different disorders [151, 152]. Presence of mitochondrial components have been found in EVs, although the exact mechanism of how mitochondria or mitochondrial components are loaded into these EVs remain elusive. A number of studies have supported the involvement of EVs in intercellular mitochondrial transfer [132, 133, 153]. The transfer of complete mitochondrial particles is a more likely episode which mediates mitochondrial function rescue via EVs during mitochondrial transfer [38]. Mitochondrial transfer can also be established via cell fusion (Fig. 3c). Spees et al. reported the fusion of human MSCs to airway epithelial cells following injury or stress [154]. Stress has shown to trigger cellular fusion as demonstrated by the fusion between transplanted bone marrow cells and cardiomyocytes following myocardial infarction [155, 156]. Cell fusion also improved rodent liver regeneration following bone marrow transplantation [157, 158]. Another probable mechanism of mitochondrial transfer is mitochondrial extrusion which allows the release of mitochondrial components or mitochondria itself under certain specific conditions [159]. This has been noted in neutrophils, basophils, eosinophils, platelets, TNF-α-mediated cell death, HeLa cells etc. [160–163].

Stem Cells for Mitochondrial Dysfunction in Stroke

Stem cell-based therapy of ischaemic stroke is although in its infancy, results from various pre-clinical and clinical trials have shown the benefits of utilising stem cells and supports its use. The mechanisms by which these stem cells elicit their effects are not yet completely understood, and before they can be translated to the bedside, a number of hurdles are required to be overcome [18]. In the previous section, we have discussed the probable mechanisms by which stem cells could transfer their healthy mitochondria to damaged cells to confer protection in different diseases. In a similar manner, transfer of mitochondria from these cells may be seen as a promising strategy to protect the brain tissue from the adverse effects of an ischaemic episode.

Babenko et al. have recently conducted a study where they have explored the transfer of mitochondria from multipotent MSCs to neural cells following the induction of mitochondrial damage [164]. This transfer, they demonstrated, was able to restore the bioenergetics of the recipient cells and also stimulated their proliferation. Similarly in a previous study conducted by Ahmad et al., they showed that mitochondrial rho GTPase 1 (Miro1) is an important protein involved in the transfer of mitochondria via the TNTs to rescue alveolar cells. Bakenko et al. also confirmed that Miro1 is involved in the transport of mitochondria from multipotent MSCs to neural cells in experimental stroke [134, 164]. Apart from the direct increase in the efficiency of mitochondrial transfer, they also showed that MSCs overexpressing Miro1 demonstrated greater potential to alleviate neurological deficit following stroke. Even in an in vitro model of ischemia (oxygen-glucose deprivation), they observed an increased transfer of mitochondria from MSCs via TNTs. Their group concluded, using mitochondria-tagged fluorescent proteins, that transfer of mitochondria took place to a larger extent via the TNTs [164].

Conclusion

The need of the hour is to look for stroke treatments that maximise recovery of neural tissue in the penumbral area in proximity to the core region, deficient in nutrients and ATP during an ischaemic attack. Mitochondria are the cell organelles that have an essential role to play in maintaining cellular survival, hence, are an important target for stroke therapy. As neurons in the penumbral region, in the absence of nutrients, start degrading in a time-dependent manner, it becomes imperative to look for strategies that could help salvage the neuronal tissue in a later stage. Stem cell therapy comes into the picture here, which in recent years has demonstrated its feasibility to protect mitochondria in numerous pre-clinical settings. Stem cells are thought to transfer mitochondria via TNTs, extracellular vesicles or simply by cellular fusion. In the coming years, researchers should focus on utilising stem cells for the therapy of ischaemic stroke and other disorders involving mitochondrial dysfunction and explore this mode of therapy to its highest potential.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflicts of interest.

Ethical Approval This article does not contain any studies with animals performed by any of the authors.

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