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## Stem cells as a therapeutic avenue for active and long-term complications of Necrotizing Enterocolitis

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### Abstract

Necrotizing enterocolitis (NEC) is a devastating neonatal intestinal disease associated with significant morbidity and mortality. Although decades of research have been dedicated to understanding the pathogenesis of NEC and developing therapies, it remains the leading cause of death among neonatal gastrointestinal diseases. Mesenchymal stem cells (MSCs) have garnered significant interest recently as potential therapeutic agents for the treatment of NEC. They have been shown to rescue intestinal injury and reduce the incidence and severity of NEC in various preclinical animal studies. MSCs and MSC-derived organoids and tissue engineered small intestine (TESI) have shown potential for the treatment of long-term sequela of NEC such as short bowel syndrome, neurodevelopmental delay, and chronic lung disease. Although the advances made in the use of MSCs are promising, further research is needed prior to the widespread use of these cells for the treatment of NEC.

### 1.0 Introduction

Necrotizing enterocolitis (NEC) is a devastating neonatal disease that is associated with high morbidity and mortality and is the leading gastrointestinal cause of death in premature infants<sup>1</sup>. NEC is characterized by abdominal distension, intestinal inflammation, microbial translocation, and systemic inflammation with potential progress to multiorgan failure and death<sup>1,2</sup>. Risk factors associated with the development of NEC include prematurity, low

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Dr. Ma is a co-founder of Persista Bio and Halomine Inc., and a scientific advisor for BeeMmunity. His roles in these companies provide no conflict to the material in this manuscript.

and very low birth weight, hyperosmolar enteral feeding, and congenital abnormalities<sup>3</sup>. Prematurity is the strongest risk factor as approximately 7% of premature neonates develop NEC, with the onset of symptoms being inversely related to gestational age<sup>3,4</sup>. The pathogenesis of NEC is not fully elucidated. However, intestinal immaturity, microbial dysbiosis, and immature intestinal and systemic immune systems are believed to contribute to the development of the disease<sup>2</sup>.

Most patients diagnosed with NEC can be treated medically with bowel rest, orogastric decompression, hemodynamic support, and broad-spectrum antibiotics. However, some patients go on to develop intestinal necrosis and perforation, requiring surgical interventions such as bowel resection or peritoneal drain placement. Mortality in this population can range from 10% to 50%<sup>4</sup>.

Necrotizing enterocolitis presents a significant challenge and is associated with numerous short-term and long-term complications. Short-term complications include wound infections, stoma issues, and intestinal strictures requiring multiple surgeries to relieve obstructions<sup>1,3,4</sup>. Following acute illness, patients are faced with potential lifelong complications including short bowel syndrome, neurodevelopmental delays, and chronic lung disease<sup>3</sup>.

There have been significant advances in NEC research over the past few decades. Numerous animal models in mice, rats, piglets, rabbits, quails, and primates have been developed and have contributed significantly to our understanding of the pathogenesis of NEC<sup>5,6</sup>. Various products such as Heparin-binding epidermal growth factor-like growth factor and probiotics have been studied as potential therapeutic agents<sup>7,8</sup>. More recently, there has been growing interest and research into the use of stem cells as novel therapeutic agents for NEC<sup>9–12</sup> (Figure 1). The purpose of this review is to 1) give a brief introduction to stem cells and their mechanism of action, 2) describe the various preclinical studies using stem cells for the treatment of NEC, and 3) review advances made in the use of stem cells for the treatment of long-term sequela of NEC.

## 2.0 Stem cells: Classification, source, and mechanism of action

Stem cells, with their seemingly endless replication and differentiation potential, have garnered significant interest as therapeutic agents in various disease processes. Mesenchymal stem cells (MSCs) are one of the most widely used and studied stem cell types. They are multipotent cells that have the potential to differentiate into different cell types including chondrocytes, adipocytes, and osteocytes,<sup>13,14</sup>. They have been shown to reduce inflammation and improve angiogenesis as well as cell and tissue recovery following ischemia<sup>15–18</sup>.

MSCs can be obtained from products of conception or adult tissues. MSCs associated with products of conception include umbilical cord blood-derived MSCs (UC-MSCs), umbilical cord tissue-derived MSCs (U-MSCs), placenta-derived MSCs (P-MSCs), and amniotic fluid-derived MSCs (AF-MSCs)<sup>15,19,20</sup>. MSCs obtained from adult tissues include adipocyte-derived MSCs (A-MSCs) and bone marrow-derived MSCs (BM-MSCs)<sup>11,15</sup>.

Different mechanisms have been proposed as to how MSCs confer their therapeutic benefits. One possible mechanism is that stem cells can migrate to the site of injury and differentiate to replace injured cells<sup>11,21</sup>. Another proposed mechanism is via the release of paracrine mediators such as hydrogen sulfide and extracellular vesicles, to reduce inflammation and enhance angiogenesis<sup>11,16,21–24</sup>.

### 3.0 Mesenchymal stem cells as therapy for necrotizing enterocolitis

#### 3.1 Stem cell therapy for active NEC

**3.1.1 Cell-based therapies**—MSCs have been studied extensively over the past decade as potential treatment options in murine models of necrotizing enterocolitis. The time of administration of stem cells during the NEC model differs in different studies<sup>15</sup>. Various studies performed administration of stem cells prior to induction of NEC as a possible preventative measure. McCulloh et al. studied the use of four different stem cells in the treatment of murine models of NEC. MSCs were isolated from various tissues and administered intraperitoneally to rat pups prior to NEC induction. The pups that received stem cells had a significantly lower incidence of NEC and decreased severity of NEC compared to controls<sup>15</sup>. They also demonstrated reduced intestinal permeability and improved gut barrier function following stem cell treatment<sup>25</sup>. Similarly, Drucker et al. performed intraperitoneal injection of UC-MSCs in mouse pups after which the pups underwent NEC induction. They reported improved clinical sickness scores and macroscopic gut injury scores in pups treated with stem cells. Zani and colleagues performed intraperitoneal injection of AF-MSCs in rat pups 24hrs after birth followed by NEC induction. They were able to show stem cell integration into the bowel wall, improved survival, lower NEC incidence, and decreased inflammation and apoptosis<sup>22</sup>.

Stem cells have also been used as a rescue therapy, with administration taking place following induction of NEC. Intraperitoneal administration of placental-derived stem cells (PSCs) in a rat NEC model after NEC induction resulted in a reduced incidence of NEC, decreased disease burden, reduced intestinal dilation and fragility, as well as improved intestinal damage with re-emergence of normal crypts on microscopic evaluation<sup>26</sup>. Similarly, BM-MSCs administration following NEC induction in rat pups has been shown to improve weight gain and clinical sickness score as well as reduce intestinal damage in microscopic evaluation<sup>27</sup>.

**3.1.2 Non-cell-based therapy**—As previously discussed, one proposed mechanism by which mesenchymal stem cells confer their therapeutic benefits is via the release of paracrine mediators such as hydrogen sulfide, cytokines, growth factors, and exosomes<sup>11,28–30</sup>. Exosomes are extracellular vesicles that contain various proteins, nucleic acids, and metabolites and have been shown to be important mediators in the function of stem cells<sup>11,28</sup>. The administration of stem cell-derived exosomes has been used as a potential cell-free therapeutic option for NEC. Exosomes isolated from MSCs and administered intraperitoneally in rat pups prior to induction of NEC demonstrated a reduction in the incidence of NEC in a dose dependent manner<sup>31</sup>. Exosomes isolated from BM-MSCs have been shown to improve gut barrier function and wound healing in murine



Bioengineering is a process by which cells, tissues, and organs are generated *de novo* to salvage organ failure and restore normal function. Therapies must restore normal anatomy and function such as absorptive and secretory functions<sup>41</sup>. Organoids are self-organized three-dimensional entities that are mainly composed of stem cells and can mimic cellular, structural, and functional aspects of a human organ<sup>50</sup>. Intestinal organoids are powerful tools in understanding the organization and function of stem cells and have been used as therapeutic agents in various disease models<sup>41</sup>. Fukuda and colleagues were able to isolate small intestine epithelial cells and culture them *in vitro* as stem cell-containing organoids. These organoids were then instilled into an EDTA-treated mouse colonic injury model. Following treatment, intestinal organoids were able to regenerate colonic epithelia<sup>51</sup>. Sugimoto et al. performed transplantation of human leucine-rich repeat-containing G protein-coupled receptor 5 (LGR5+) colonic organoids into a murine colonic injury model. They observed that xenografted organoids were multipotent, able to form crypt structures, and had self-renewal capabilities<sup>52</sup>. Furthermore, transplantation of LGR5+ colonic organoids into damaged mouse colon has been shown to result in the integration of donor cells with the regeneration of single-layered epithelium, self-renewing crypts with normal function and histology, and long-term engraftment<sup>53</sup>. Cortez and colleagues generated human intestinal organoids from embryonic stem cells and transplanted them into immunocompromised mouse mesentery. They demonstrated successful engraftment and growth of intestinal organoids along vascular roots with histology demonstrating major cell types such as enterocytes, endocrine cells, and Paneth cells<sup>49</sup>.

In addition to direct transplantation, intestinal organoids have been used to generate tissue engineered small intestine (TESI). Grant et al. were able to generate

TESI from human and mouse intestinal organoids. Additionally, they demonstrated that TESI could grow with epithelium-facing lumen with tight junctions, ion channels, brush border enzymes, and microvilli. Supporting mesenchyme, muscle, and progenitor cells were also present<sup>54</sup>. Grikscheit and colleagues isolated small intestine organoid units from rat pups and generated TESI using a polyglycolic acid (PGA) scaffold. TESI was then transplanted into rats by externalizing and wrapping the greater omentum around the TESI construct. Four weeks post-transplantation, TESI+ animals and controls underwent approximately 80% of bowel resection with end-to-end anastomoses. Following initial weight loss in both TESI+ and TESI- groups, TESI+ animals were able to gain weight up to 98% of their preoperative weights. Additionally, TESI+ animals had higher serum B12 levels and showed appropriate intestinal architecture<sup>55</sup>.

Supplementation of human pluripotent stem cell (hPSC) derived TESI with hPSC derived enteric neural crest cell has demonstrated the establishment of submucosal and myenteric ganglia and neuron-dependent contractility and relaxation<sup>56,57</sup>. Kitano et al. performed ex-vivo vascularization of hPSC-derived TESI with human endothelium. Following transplantation into immunodeficient rats, intestinal epithelium survival and maturation were appreciated as well as the absorption of glucose and fatty acids<sup>58</sup>. Although organoid and TESI-based treatment for short bowel syndrome is a distant therapeutic goal, pre-clinical animal studies have been promising in generating intestinal epithelium with normal architecture and function.

**3.2.2 Neurodevelopmental delay**—Neurologic injury and neurodevelopmental delay are well documented long-term sequela of NEC<sup>3,59</sup>. The systemic inflammatory response, as well as systemic hypotension associated with NEC, are believed to contribute to white-matter injury which may lead to a higher incidence of cerebral palsy, cognitive deficits, and visual and hearing impairment<sup>60–63</sup>. Animal models have further solidified the adverse relationship between NEC and neurological injury. Biouss et al. studied the neurological changes that occur in mouse pups following NEC induction. Mouse pups undergoing NEC had smaller brain weights, thinner cortices, increased levels of apoptosis, lower number of neurons, myelinating oligodendrocytes and neural progenitor cells, increased pro-inflammatory cytokines, and increased activated microglia<sup>64</sup>.

NEC has been shown to be a risk factor for the development of antenatal brain injury<sup>61,63</sup>. Although there has not been a study that specifically looks at the use of MSCs to address the neurologic injury seen in NEC, there are various studies that investigate the use of MSCs to address antenatal brain injury such as hypoxic-ischemic encephalopathy (HIE) and stroke<sup>42,65</sup>.

Opplinger and colleagues studied the use of UC-MSCs for the treatment of preterm white matter injury. They performed intranasal administration of UC-MSCs in rat pups that underwent brain injury by combined hypoxic-ischemic and inflammatory insult and were able to demonstrate increased myelination in the stem cell treated group compared to controls<sup>66</sup>. UC-MSCs have also been studied in a rat pup model of ischemia-induced severe brain injury. Intraventricular transplantation of UC-MSCs showed improved body weight gain, decreased infarct volume, and improved functional test and histologic abnormality<sup>67</sup>.

Intranasal administration of UC-MSCs in rat pups with hypoxic-ischemia brain injury significantly reduced loss of brain tissue and markers of neuroinflammation<sup>43</sup>. Similarly, intraventricular transplantation of UC-MSCs in a murine model of HIE demonstrated significant improvement in markers of severe HIE such as increased brain infarction, increased cerebral spinal fluid cytokine levels, and impaired functions<sup>68</sup>. Finally, Chen et al. used rat pups to induce periventricular white matter injury using an intracerebral injection of ibotenic acid on postnatal day 5. They then transplanted MSCs near the lesion and showed migration of MSCs to the injury site and improved sensorimotor function<sup>69</sup>.

**3.2.3 Chronic lung disease**—Premature infants are at risk for significant respiratory distress and bronchopulmonary dysplasia (BPD)<sup>4</sup>. BPD is a chronic lung disease that is associated with various such as prematurity, inflammation and infection, mechanical ventilation, and oxygen toxicity<sup>70</sup>. Historically, BPD was associated with prominent fibrosis, airway smooth muscle hypertrophy, and pulmonary hypertension. With the advancement in the care of premature infants and the use of surfactants, patients have improved histological features and marginally improved clinical outcomes<sup>71</sup>. However, BPD remains a significant cause of morbidity and mortality in premature infants.

Patients with NEC, particularly surgical NEC, are more likely to develop respiratory distress while various respiratory managements of premature neonates have been suggested as potential risk factors for the development of NEC<sup>72,73</sup>. The gut-lung axis has

been described as a potential explanation for the association seen between intestinal and respiratory pathologies<sup>72</sup>. The microbial dysbiosis and translocation that occur in necrotizing enterocolitis causes systemic inflammation that likely contributes to neonatal lung injury<sup>72,74</sup>. Preclinical animal studies have demonstrated that toll-like receptor 4 activation in mice undergoing an intestinal ischemia/reperfusion model resulted in lung injury<sup>75</sup>. Various clinical studies have also demonstrated the association between NEC and BPD. A multicenter cohort study from Spanish Neonatal Network investigated preterm infants < 32wks gestational age at birth and found that patients with surgical NEC had an odds ratio of 2.0 of developing BPD<sup>76</sup>. Additionally, a meta-analysis of five randomized control trials studying low (85–89%) vs high (91–95%) oxygen saturation targets in premature infants found that infants in the lower oxygen saturation target group had a higher incidence of severe necrotizing enterocolitis<sup>77</sup>.

Pierro et al. studied the use of umbilical cord blood-derived MSCs in a rat pup model of BPD. Following intratracheal administration of MSCs prophylactically or as treatment following lung injury, groups that were treated with MSCs had improved lung compliance, preserved alveolar growth, and restoration of alveolar architecture<sup>44</sup>. Similarly, intravenous administration of MSCs and MSC-conditioned media in a mouse BPD model demonstrated an increase in bronchioalveolar stem cells which are adult lung stem cells capable of differentiation and self-renewal<sup>78</sup>.

## 4.0 Future Directions

### 4.1 Stem cell encapsulating and packaging devices

Cell encapsulation was first reported in the 1930s<sup>79,80</sup>. Research into islet cell encapsulation and delivery for the treatment of diabetes has thus far been the main focus of cellular encapsulation and delivery research<sup>80,81</sup>. However, over the past few years, stem cell encapsulation has garnered new interest<sup>82,83</sup>. As mentioned earlier, studies have demonstrated that stem cells can elicit a robust immune response upon administration<sup>84</sup>. This makes stem cells susceptible to host immune responses and limits their long-term benefit<sup>85</sup>. Furthermore, stem cells, particularly induced pluripotent stem cells, have been associated with increased tumorigenicity<sup>86–88</sup>. Therefore, a cell encapsulation and delivery method has a promising future in stem cell therapy because it can 1) limit the dissemination of cells while allowing the exchange of paracrine mediators, 2) reduce the risk of immune activation and the need for immunosuppression, 3) protect therapeutic cells from host defenses potentially giving longevity to implanted cells, and 4) allow retrievability following resolution of disease<sup>80</sup>.

Hydrogel macro and microencapsulation devices have been studied over the past decades for their ability to house and deliver cell-based therapies for the treatment of various diseases<sup>80,89</sup>. Yang et al. synthesized an injectable alginate hydrogel that can retain UC -MSC derived exosomes to repair bone defects in rats and demonstrated successful migration and differentiation of an osteoblast cell line<sup>90</sup>. Li et al. packaged hPSC-derived exosomes into chitosan hydrogel and studied their stability and therapeutic effect. They demonstrated that exosome proteins and microRNAs remained stable while packaged inside hydrogel and maintained their therapeutic effect during a murine hindlimb ischemia

model<sup>91</sup>. Wang et al. used a nanofibrous encapsulated device to package stem cell-derived islet  $\beta$  cells and demonstrated containment of cells within the device, reversal of diabetes in mice, and retrievability of the device weeks following implantation<sup>92</sup>. Despite the promising results, fibrotic reactions against the encapsulation materials and insufficient supply of oxygen to the encapsulated cells have been identified as two key challenges that must be overcome for future clinical applications of this approach. Furthermore, the ideal time and route of administration, length of therapy, and the ability to monitor transplanted cells are yet to be fully elucidated.

## 4.2 Gut-on-chip

As research into the pathophysiology and therapeutics of necrotizing enterocolitis and other intestinal pathologies continue, finding reproducible and realistic disease models are necessary. As mentioned earlier, various animals have been used to study necrotizing enterocolitis, its pathogenesis, and potential therapies<sup>5,93</sup>. However, animal models are costly as well as time and labor intensive<sup>5,6</sup>. With the fundamental three “R”s (replacement, reduction, and refinement) of animal research in mind, there has been continued research into two-dimensional and three-dimensional gut-on-chip models to study various gastrointestinal diseases<sup>94</sup>. Induced pluripotent stem cells have been used to generate three-dimensional gut-on-chip models<sup>95</sup>. Gut-on-chip models use a semipermeable membrane to separate two chambers allowing epithelial cells growing on this membrane to have apical and basolateral sides. Nutrition supply and waste removal are achieved by a continuous flow of culture media<sup>95,96</sup>. These models have been used to test the pharmacokinetics of drugs as well as molecular interactions between microbiome and hosts<sup>95,97–99</sup>. Furthermore, three-dimensional gut-on-chip has been used to study intestinal absorption, gut-barrier function, intestinal inflammation, and host-microbiome interactions<sup>100–103</sup>.

## 4.3 Clinical applications of stem cells

According to [ClinicalTrials.gov](https://clinicaltrials.gov), there are over 1,100 ongoing and completed clinical trials involving mesenchymal stem cells<sup>104</sup>. There are 21 studies registered for the use of MSCs in BPD<sup>105</sup> and over 30 clinical trials using MSCs for stroke and HIE<sup>106,107</sup>. To date, there has only been one clinical application of MSCs in the treatment of NEC. Akduman and colleagues reported a case of a 37wk neonate who developed NEC following a recurrent episode of supraventricular tachycardia. The patient progressed to requiring surgical intervention with bowel resection and ostomy creation. On postoperative day 4, the patient underwent intravenous administration of UC-MSCs. Following this, the patient had improved mesenteric blood flow and was able to start enteral feeds eight days following stem cell administration and progress to ostomy takedown on postoperative day forty-six<sup>108</sup>.

As mentioned earlier, patients with NEC have a higher likelihood of developing various neurodevelopmental impairments such as cerebral palsy and cognitive, hearing, and visual impairments<sup>109</sup>. Several clinical trials investigate the use of stem cells to address different neurological injuries. Few trials study the use of cord blood in children with HIE and cerebral palsy<sup>110</sup>. Cord blood has a high concentration of stem cells such as umbilical cord-derived mesenchymal stem cells, somatic stem cells, endothelial progenitor cells, and embryonic-like stem cells<sup>110</sup>. A meta-analysis of multiple randomized controlled trials and

clinical trials of stem cell use for cerebral palsy demonstrated the safety and efficacy of stem cells of various origins in improving short-term motor skills in patients with CP<sup>110,111</sup>.

The first clinical trial to use MSCs for the treatment of BPD was performed in South Korea in 2010<sup>112</sup>. Nine premature and low birth-weight infants requiring mechanical ventilation underwent intratracheal administration of MSCs. No significant adverse effect was seen with MSC treatment or route of administration in short-term (84 days) and long-term (2 years) follow-ups. Furthermore, infants who received MSCs had a lower incidence of BPD and reduced inflammatory markers<sup>112</sup>. It is important to note that there were no control groups for this study. However, this study was seminal and opened the door for subsequent clinical trials using MSCs in BPD<sup>70,113</sup>.

## 5.0 Ethical considerations

Using stem cells for research and treatment can raise ethical and legal considerations. The timing and source of stem cell harvest, potential tumorigenicity of cells, as well as the potential downstream ramification of transplanted allogenic stem cells, remain areas of concern<sup>114,115</sup>.

As described earlier, stem cells can be derived from various sources. Since the first human embryonic stem cell (ESC) was isolated in 1998, there has been considerable debate on the ethical implications of stem cell research and therapy<sup>115</sup>. As a result, the National Academy of Science established a committee in 2003 to help guide researchers and institutions<sup>115</sup>. The committee has created guidelines to address multiple areas of concern, including establishing oversight, obtaining consent from donors, and defining acceptable and non-acceptable categories of research<sup>115,116</sup>. Adult tissue-derived stem cells, such as A-MSCs or BM-MSCs, and products-of-birth derived stem cells, such as UC-MSCs and U-MSCs, have fewer ethical concerns than ESCs<sup>114</sup>.

The controversies surrounding stem cell research, particularly ESCs, led researchers to investigate ways to induce differentiated somatic cells to become pluripotent stem cells<sup>114,115</sup>. Induced pluripotent stem cells (iPSCs) have been studied over the years as potential replacements for naturally occurring stem cells<sup>117,118</sup>. However, various studies have raised the concern for the safety of these cells as the de-differentiation process has been shown to cause genetic and epigenetic modifications<sup>118,119</sup>. Additionally, hESCs have been shown to acquire chromosomal abnormalities during in vitro cultures<sup>119</sup>. Furthermore, the extent of the immune response that could be elicited by allogeneic transplantation of stem cells is not fully understood<sup>84,85</sup>. This raises safety concerns for the potential introduction of tumorigenic and immune system-activating cells during therapy. To this end, researchers sought to find an ideal route and method of stem cell administration and recovery leading to the advent of cell encapsulating devices, as discussed above.

Although there have been significant advances in stem cell research, much remains unanswered when it comes to widespread application and therapeutic use. Therefore, a clear understanding and communication of expectations and shortcomings of stem cell therapy

should be clearly outlined during the consent process to allow informed decision-making and the safety of patients.

## 6.0 Conclusion

Necrotizing enterocolitis is associated with significant morbidity and mortality in neonates. Mesenchymal stem cells have emerged as potential therapeutic agents. They have shown promise as treatment options for acute NEC as well as the various long-term sequela of NEC (Figure 1). Further research is needed to determine safety, efficacy, and mode of administration prior to broad clinical application.

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## Biography

Linseigh Green survived necrotizing enterocolitis in 1997 thanks to a sigmoidectomy. As she grew older, she began to suffer from an increasing number of long term gastrointestinal and neurological complications. Realizing that many of her counterparts were dealing with more severe challenges, at the age of 21, Linseigh began raising awareness about NEC's impact on survivors' futures, and helped lead the first known study on the subject. She now works as the NEC Society's Outreach Manager. Though Linseigh's parents were told she'd never succeed in school, she has graduated from three university programs with top honors, including Cambridge in the UK.



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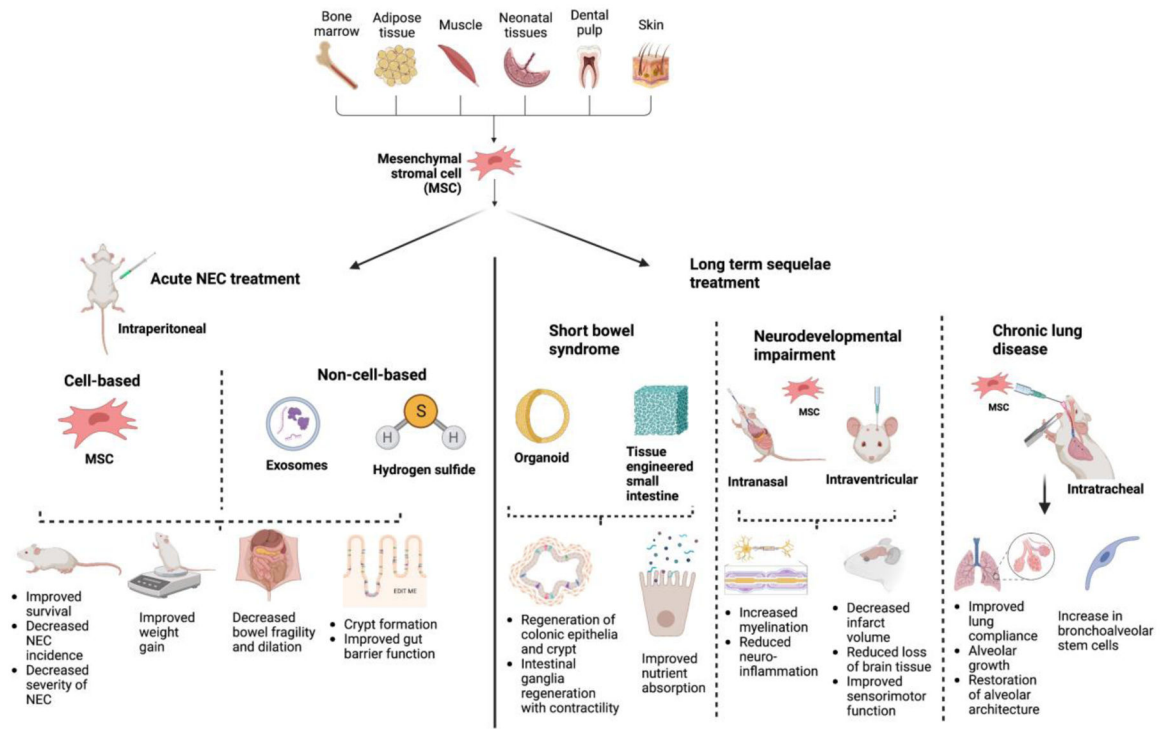
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**Figure 1:**  
Mesenchymal stem cells in the treatment of NEC-summary of preclinical studies