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AIMS AND SCOPE

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EDITORIAL

Enhancing the functionality of mesenchymal stem cells: An attractive treatment strategy for metabolic dysfunction-associated steatotic liver disease?

Xiao-Qian Shan, Lan Zhao

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Abstract

The intrinsic heterogeneity of metabolic dysfunction-associated fatty liver disease (MASLD) and the intricate pathogenesis have impeded the advancement and clinical implementation of therapeutic interventions, underscoring the critical demand for novel treatments. A recent publication by Li *et al* proposes mesenchymal stem cells as promising effectors for the treatment of MASLD. This editorial is a continuum of the article published by Jiang *et al* which focuses on the significance of strategies to enhance the functionality of mesenchymal stem cells to improve efficacy in curing MASLD, including physical pretreatment, drug or chemical pretreatment, pretreatment with bioactive substances, and genetic engineering.

Key Words: Metabolic dysfunction-associated fatty liver disease; Mesenchymal stem cells; Preprocess; Cell survival; Therapeutic strategy

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Core Tip: Metabolic dysfunction-associated fatty liver disease is a serious health challenge, and new therapies are urgently needed. Jiang *et al* proposed mesenchymal stem cells as a promising therapeutic approach for metabolic dysfunction-associated fatty liver disease, emphasizing the targeting of key molecular pathways such as glycolipid metabolism, inflammation, oxidative stress, endoplasmic reticulum stress, and fibrosis. This paper concurs this opinion and further discusses some strategies enhance functionality mesenchymal stem cells.

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INTRODUCTION

As a chronic liver disease, metabolic dysfunction-associated fatty liver disease (MASLD) affects approximately one-third of the global population[1]. The rising prevalence of MASLD is associated with an array of comorbidities, including type 2 diabetes mellitus, cardiovascular disease, obesity, and insulin resistance. The precise pathogenesis of this disease remains unclear. Previous studies have indicated that the two-hit theory may provide an explanation for the pathogenesis of this disease[2]. Firstly, the accumulation of lipids and the development of insulin resistance result in a reduction in the degradation of fatty acids, which in turn leads to the formation of hepatic steatosis. Subsequently, the liver becomes susceptible to inflammatory cytokines, adipokines, mitochondrial dysfunction, and oxidative stress. Based on these factors, reactive oxygen species is triggered to increase, inducing fatty hepatitis, fibrosis, and cirrhosis.

However, as research progresses, it becomes evident that this theory is unable to fully summarize the pathogenesis of the disease. Despite the preclinical studies of this disease involving multiple targets, few drugs have been applied to the clinic due to the complexity of its pathogenesis. Mesenchymal stem cells (MSCs) are adult pluripotent cells with high self-renewal, multidirectional differentiation, good biocompatibility, low immunogenicity, and paracrine function. The pleiotropic effects of MSCs are potentially more advantageous relative to drug therapy and have received much attention in the study of innovative therapeutic approaches for liver disease. Recently, Jiang *et al*[3] presented the same study in the *World Stem Cell Journal*. We agree with the authors' findings that MSCs and paracrine secretions exhibited regulation of glycolipid metabolism, anti-inflammatory, anti-apoptotic, amelioration of oxidative stress, anti-fibrotic, and stimulation of angiogenesis and tissue regeneration in damaged liver in different experimental models of liver diseases. However, a key issue in the clinical application of MSCs is how to maintain their stability and efficacy after systemic administration. Current stem cell preconditioning protocols allow to improve their therapeutic efficacy to overcome the limitations of MSCs, *i.e.*, migration rate, survival, implantation and paracrine activity. Therefore, we discuss strategies to enhance the effectiveness and stability of MSCs in the treatment of liver disease, including physical pretreatment, drug or chemical pretreatment, pretreatment with bioactive substances, and genetic engineering (Figure 1).

STRATEGIES TO ENHANCE THE THERAPEUTIC POTENTIAL OF MSC

Physical pretreatment

Physical preconditioning is the most prevalent method of manipulation of MSCs to counteract the harsh microenvironment (improve survival and function after transplantation). Among them, hypoxia initiation is the more studied treatment operation. Compared to normoxic cultured MSCs, oxygen solubility *in vivo* and oxygen content in liver tissue and circulatory system were extremely low. MSCs amplification is difficult to adapt to drastic oxygen pressure changes. Short-term hypoxic exposure upregulates Sug1 and downregulates HSP90α levels, resulting in increased immunogenicity of MSCs[4,5]. Hypoxia or hypoxia and reoxygenation have been shown to acclimatize MSCs to changes in oxygen in defined microenvironments *in vitro* while promoting their pluripotency and enhancing their cellular activity and therapeutic efficacy[6]. Hypoxia initiation increased the expression of mediators of anti-inflammatory and regenerative responses in MSCs [including interleukin (IL)-6, tumor necrosis factor-alpha, hepatocyte growth factor, vascular endothelial growth factor, Jagged 1 and prostaglandin E synthase], promoting liver regeneration and inhibiting hepatocyte death[7-9]. Hypoxic priming also prevents MSCs senescence by upregulating the polypyrimidine tract-binding protein 1/phosphoinositide 3-kinase-mediated autophagy and downregulating p16INK4A, p53, and p21[10,11].

Other physical methods used for triggering include three-dimensional culture, electrical stimulation, serum deprivation preconditioning, and more. Compared to the traditional two-dimensional culture of bone marrow MSC, the three-dimensional porous scaffold structure increases the surface area for cells to interact with the extracellular matrix. In addition, the three-dimensional culture more closely resembles the conditions of the natural extracellular matrix, which provides a better environment for cell attachment and growth, resulting in a substantial increase in MSCs yield[12]. Electrical stimulation of MSC enhanced their proliferation, migration, differentiation and scaffold adhesion[13]. In addition, it has been found that simultaneous pretreatment of MSCs with oxidative and serum deprivation stress greatly improves their survival under adverse conditions and increases the stability of the graft[14]. In conclusion, most studies have demonstrated that physical pretreatment improves the outcome of MSCs therapy for liver disease. It has the potential to be safely to translated into the clinic.

Drug or chemical pretreatment

In vitro, pretreatment of MSCs with drugs or chemicals enhances the therapeutic efficacy of MSCs in liver diseases by repairing the lost functions through modulation of various pathways. Among them, oxidative stress and inflammation are important factors affecting the survival of transplanted MSCs[15]. MSCs pretreated with curcumin significantly downregulated the *ASK-JNK-BAX* gene expression involved in mitochondrial stress and apoptosis[16]. And, curcumin

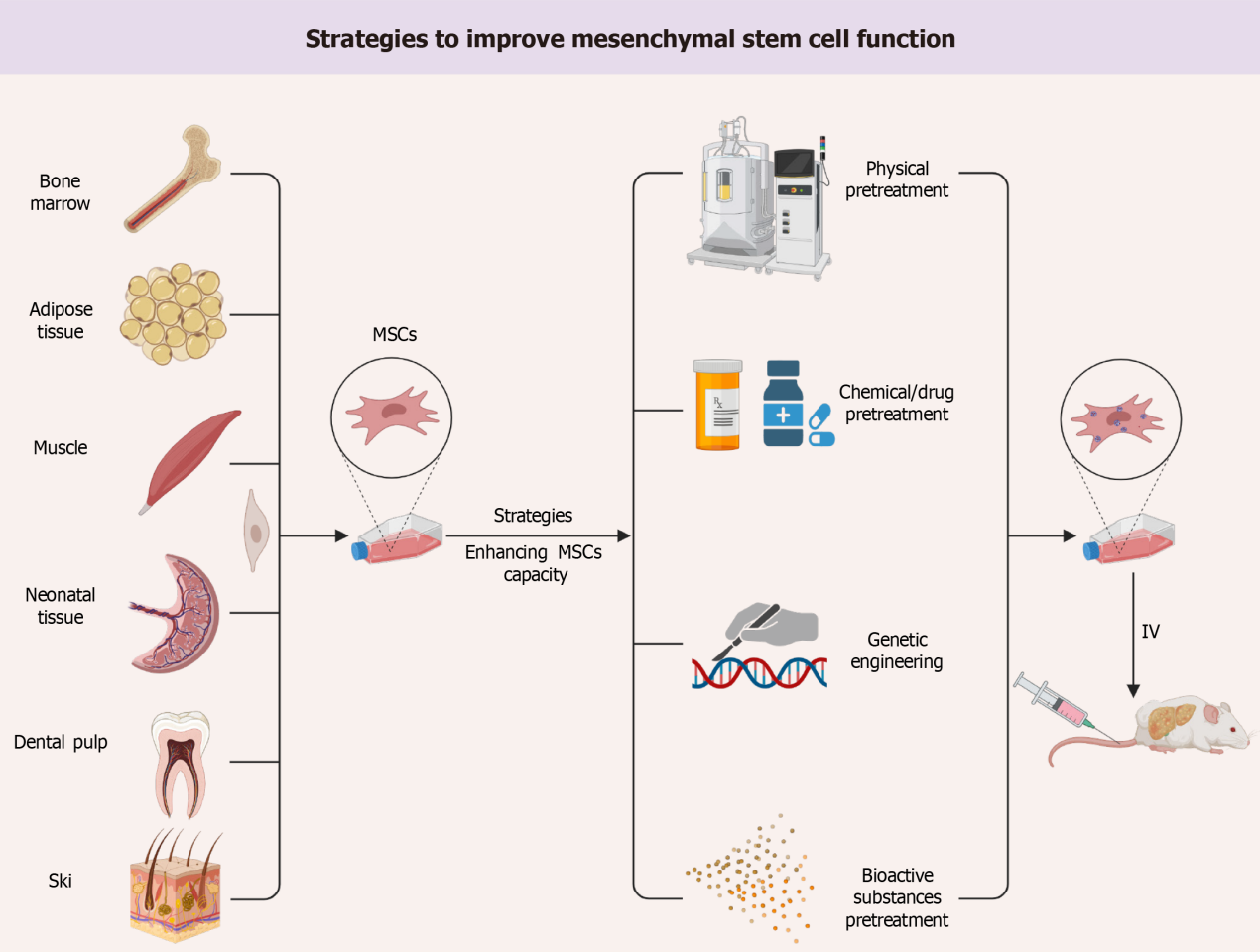

Figure 1 Strategies to improve mesenchymal stem cell function. MSC: Mesenchymal stem cell; IV: Intravenous injection.

pretreated MSCs improved liver fibrosis and prevented MASLD recurrence compared to MSCs[16]. Similarly, baicalin-pretreated MSCs ameliorated liver injury by activating p62/Kelch-like ECH-associated protein 1/nuclear factor-erythroid 2-related factor 2 signaling and inhibiting oxidative burst, inflammation, and lipid peroxidation-induced iron death[17]. Eugenol pretreated MSCs inhibited hepatic fibrosis and promoted hepatocyte regeneration and survival by inhibiting the inducible nitric oxide synthase pathway and modulating the transforming growth factor (TGF)-β/small mother against decapentaplegic pathway[18-20]. In addition, several antioxidant drugs and anti-inflammatory agents have been used to overcome oxidative stress and inflammation thereby improving MSCs survival *in vitro*. For example, low-dose antioxidant (reduced glutathione and melatonin) treatments were able to inhibit reactive oxygen species generation thereby maintaining stemness and multidirectional differentiation potential during long-term *in vitro* passaging[21]. One study using NIR-II fluorescent nanoparticles to track MSCs viability also confirmed that glutathione (antioxidant) and dexamethasone (anti-inflammatory) improved stem cell implantation efficiency and enhanced MSCs for liver fibrosis[22]. Another tracking experiment showed that more vitamin E-pretreated MSCs were transplanted into rat livers[22]. Vitamin E pretreated MSCs reduced the expression of oxidative stress-related genes (*Cyp2e1*, *Hif1-alpha*, and *Il-1beta*) as well as liver fibrosis-related gene markers (*Tgf-beta1*, *alpha-Sma*, and *Col1alpha1*), which prevented Carbon tetrachloride 4-induced oxidative stress, and improve their tolerance to unfavorable ecological niches in fibrotic livers[23]. Considering that some antioxidant and anti-inflammatory agents (*e.g.*, vitamin E and reduced glutathione) have been used clinically in patients with MASLD, drug or chemical pretreatment is a promising clinical strategy.

Bioactive substance pretreatment

Initiating MSCs with bioactive substances is also one of the pretreatment strategies to improve their function involving growth factors, cytokines, trophic factors, hormones, vitamins, and others[15]. Compared to unmodified MSCs, TGF-β1 pretreated MSCs showed maximal inhibition of TGFβ-small mother against decapentaplegic 2/3 signaling and expression of fibrosis markers (E-cadherin, α-smooth muscle actin, type I collagen-alpha 1) in activated hepatic stellate cells[24]. Zhang *et al*[25] found that using interferon-γ for priming MSCs promoted indoleamine 2,3-dioxygenase (IDO) secretion which leads to better therapeutic effects in the liver injury model. On the one hand, IDO enhanced the AMP-activated protein kinase-mechanistic target of rapamycin autophagy axis and induced protective autophagy in hepatocytes; on the other hand, IDO increased Treg counts and boosted MSCs' ability to induce immune tolerance[16]. In addition, tumor necrosis factor α[26,27], interleukin(IL)-1[27,28], vitamin E[22], melatonin[21], and oxytocin hormones[29] can enhance cell proliferation, survival, migration, and homing of MSCs by inducing signaling pathways such as mitogen-activated

protein kinases, mechanistic target of rapamycin, protein kinase B/extracellular signal-regulated kinase 1/2, nuclear factor-kappaB and IL-1R1/myeloid differentiation primary response 88.

Genetic engineering

The homing of MSCs is primarily the result of interactions between ligands and receptors. Correcting or modifying the gene expression of receptors/ligands on MSCs is a potential way to increase homing rates within target tissues. MSCs were modified by overexpression of migration-related genes [CC chemokine receptor 2, chemokine receptor 4, chemokine (C-X-C motif) ligand 9 and mesenchymal-epithelial transition factor] to enhance their homing ability. Overexpression migration related genes [CC chemokine receptor 2, chemokine receptor 4, chemokine (C-X-C motif) ligand 9 as well as mesenchymal-epithelial transition factor] have been used to enhance MSCs mobilization, homing and engraftment[30]. MSCs genetically modified with protein kinase B 1 have a survival advantage and stronger immunomodulatory effect both *in vitro* and *in vivo*, thus suggesting the therapeutic potential for amelioration of liver injuries[31]. Another editing method is by promoting exosome overexpression in MSCs thereby enhancing their reproduction and homing and treatment. For example, overexpression of miR-27b can inhibit directional migration of primary cultured chemokine receptor 4-positive murine MSCs by down-regulating stromal cell-derived factor-1α levels[32]. In conclusion, these genetic engineering strategies hold great promise for enhancing MSCs homing, survival, and therapeutic capabilities.

CONCLUSION

Currently, stem cell-based therapies are showing promise in both preclinical and clinical settings. However, the field is still evolving and further research will be required in the future before it can be used as a conventional treatment modality for MASLD. The first challenges addressed are MSC transplantation use and long-term therapeutic outcomes. Pretreatment of MSCs by physical pretreatment, drugs and chemicals, bioactive substances, and gene modification prior to administration improves the *in vitro* and *in vivo* function of MSCs, thereby highly enhancing the repairing efficacy for liver disease models. These modified MSCs will be more promising than cells from naive MSCs.

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Bioengineering breakthroughs: The impact of stem cell models on advanced therapy medicinal product development

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Abstract

The burgeoning field of bioengineering has witnessed significant strides due to the advent of stem cell models, particularly in their application in advanced therapy medicinal products (ATMPs). In this review, we examine the multifaceted impact of these developments, emphasizing the potential of stem cell models to enhance the sophistication of ATMPs and to offer alternatives to animal testing. Stem cell-derived tissues are particularly promising because they can reshape the preclinical landscape by providing more physiologically relevant and ethically sound platforms for drug screening and disease modelling. We also discuss the critical challenges of reproducibility and accuracy in measurements to ensure the integrity and utility of stem cell models in research and application. Moreover, this review highlights the imperative of stem cell models to align with regulatory standards, ensuring using stem cells in ATMPs translates into safe and effective clinical therapies. With regulatory approval serving as a gateway to clinical adoption, the collaborative efforts between scientists and regulators are vital for the progression of stem cell applications from bench to bedside. We advocate for a balanced approach that nurtures innovation within the framework of rigorous validation and regulatory compliance, ensuring that stem cell-base solutions are maximized to promote public trust and patient health in ATMPs.

Key Words: Stem cells; Advanced therapy medicinal products; Tissue-engineered products; Health; Three-dimensional cell culture

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Core Tip: Stem cells play a crucial role in tissue engineering by offering the potential for regenerating of damaged tissues, which is critical for developing advanced therapy medicinal products. Stem cells can differentiate into specific cell types and promote tissue repair through various mechanisms. When combined with tissue engineering techniques, stem cell therapy enhances cell viability, differentiation, and therapeutic efficacy, overcoming disease treatment limitations. However, translating stem cell research into approved clinical therapies has been challenging. Regulatory bodies have provided guidelines to ensure the safety and efficacy of advanced therapy medicinal products utilizing stem cells before the approval for clinical use.

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INTRODUCTION

Advanced therapy medicinal product (ATMP) was defined by European Union (Directive 2001/83/EC) and amended by the ATMP Regulation (EC No 1394/2007), whereas, in the United States, the term used is cellular and gene therapy products. In this review, we will adopt ATMP. These products represent a significant advancement in medical treatment, focusing on innovative therapies for new regenerative approaches for well-know or rare diseases/conditions (orphan and unmet needs)[1]. These biological products include three main categories: Gene therapy, somatic cell therapy, and tissue-engineered products (TEPs), all of which aim to treat the root causes of diseases rather than just their symptoms. Stem cells can differentiate into multiple cell types and secrete trophic factors, making them an attractive tool for ATMPs, revolutionizing the field of regenerative medicine. New three-dimensional (3D) cell culture techniques have enhanced cell properties relevant for tissue regeneration, such as cell viability, differentiation, and secretion of pro-regenerative factors, overcoming the limitations of stem cell therapy alone for organ replacement in the tissue engineering concept. In this review, we discuss the current use of stem cells in ATMP development, specifically, for TEP and the regulatory landscape worldwide.

ATMPs

ATMPs represent a diverse category of medicinal products based on, or the combination of them and the addition of medical devices. ATMPs are medicines that are based on the manipulation of biological materials (genes, cells, and/or tissues) and combined with medical devices to achieve therapeutic effects (Figure 1). The three main categories include: (1) Gene cell therapy medicine (GCTM)- which involves the introduction, removal, or alteration of genetic material within a patient's cells to treat or prevent disease; (2) Somatic cell therapy medicine (SCTM) - this therapy transfers genetic material into somatic (non-reproductive) or stem cells to treat diseases, ensuring that future generations do not inherit the changes; and (3) TEP: This therapy contain engineered cells or tissues designed to regenerate, repair, or replace damaged human tissues. Clinical use of SCTM and TEP is referred as regenerative medicine.

Recent reports show numerous ongoing clinical trials for ATMPs, with a significant proportion in the early development phases. These trials predominantly focus on oncology, genetic disorders, cardiovascular and musculoskeletal diseases. The complexity of ATMPs often necessitates innovative trial designs, including small sample sizes and adaptive methodologies to accommodate the unique characteristics of these therapies[2].

A recent study assessed the efficacy of ATMPs in healing long bone delayed unions and non-unions through clinical and radiological consolidation at 3, 6, and 12 months of the initial fracture[3]. Clinical consolidation occurred earlier, while radiological consolidation reached 92.8% at 12 months. Bone biopsies confirmed bone formation around bioceramic granules, with better consolidation in non-smokers and slight delays in tibial non-unions. The study showed effective bone healing using expanded human bone marrow mesenchymal stem cells (MSCs) with biomaterials, though consolidation rates were lower in smokers.

However, ATMPs come with a number of known and unknown risks, many of which are unique to this product class. Some of the main risks associated with ATMPs include: (1) Related to the novel mechanisms of action (may cause new risks to patients due to their novel mechanisms of action); (2) Related to manufacturing complexity (ATMPs present a high degree of technical complexity and substantial challenges to their manufacture and risks are related to improper handling, post-release of the product and prior to its use, have the potential to impair the quality and safety of the product as well as increase risks associated with the production process); (3) Related to extensive manipulation (products subjected to substantial manipulation in the laboratory or that perform a function in the recipient that is different from

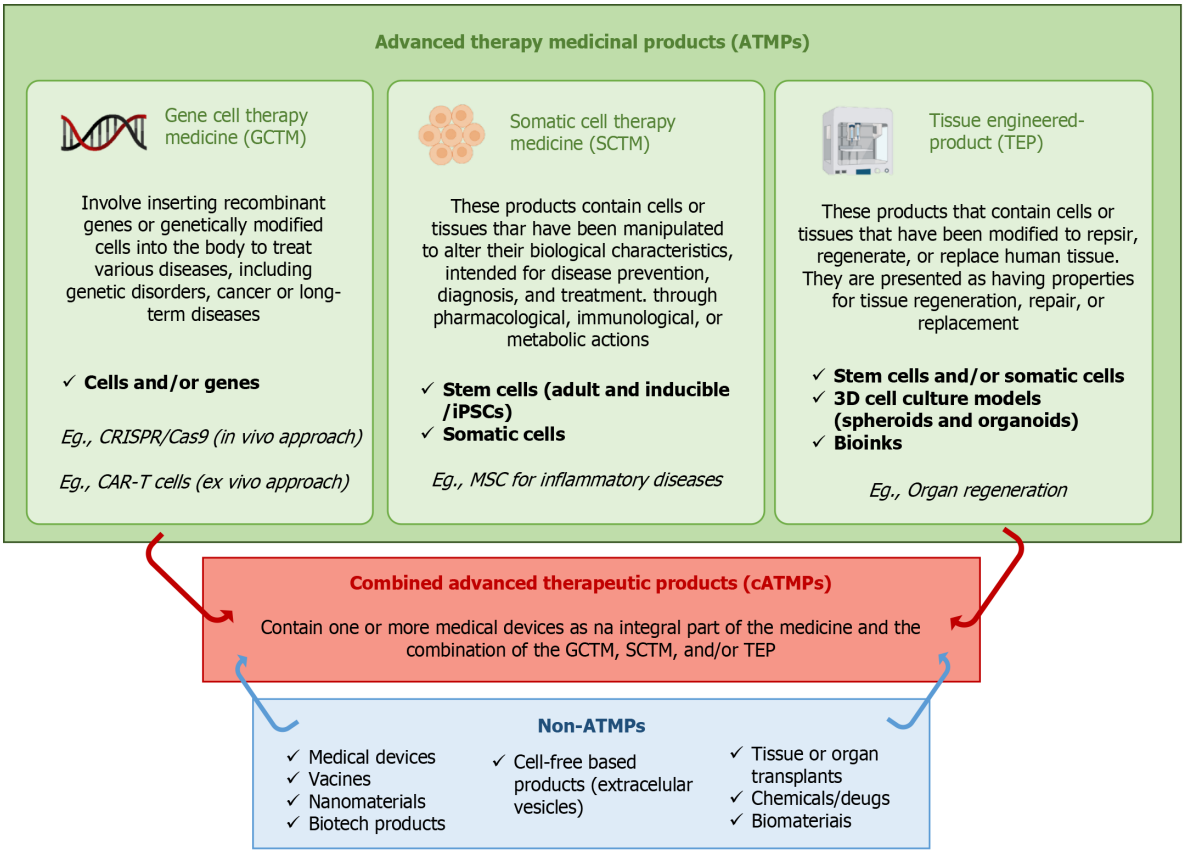

Figure 1 Three main types of advanced therapy medicinal products. Non-advanced therapeutic products are regulated separately from advanced therapeutic products and may be used in combination with advanced therapeutic products. ATPs: Advanced therapeutic products; cATMP: Combined advanced therapy medicinal product; SCTM: Somatic cell therapy medicine; GTMP: Gene therapy medicinal product; TEP: Tissue-engineered products; iPSC: Induced pluripotent stem cell; MSC: Mesenchymal stem cell; CRISPR/Cas9: Clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9; CAR-T: Chimeric antigen receptor T; 3D: Three-dimensional.

the function performed in the donor pose a high intrinsic risk to health and the degree of manipulation to which the cells have been subjected has more impact on risk assessment than the origin of the cells - autologous or allogenic); and (4) Related to improper administration (the detailed description of the conditions of use of the ATMP must be carefully elaborated and informed by the manufacturer to the person in charge of the use/application of the product and should be carried out only in authorized specialized centers). To mitigate these risks, a flexible approach to risk identification, evaluation and mitigation is needed, considering all areas of development including the biological activity, quality attributes, manufacturing process steps and therapeutic administration procedures. Appropriate risk minimization measures, such as specialized trainings for physicians and targeted educational materials, may also be necessary[2,4,5].

Stem cells in ATMPs

Over time, advancements in stem cell research have led to the development of cell-based therapies to address diseases resistant to conventional treatments[6]. A significant milestone leading to these advancements was the breakthrough in stem cell cultivation, which led to the discovery of human embryonic stem cells (ESCs) and the development of xeno-free culture systems[7]. These foundational advancements laid the groundwork for harnessing the therapeutic potential of stem cells, shaping the trajectory of regenerative medicine. By harnessing the regenerative capacity of stem cells, researchers can overcome limitations in current treatment modalities, paving the way for personalized therapies. ATMPs utilize a variety of cells and tissues, and their specificity directly affects their effectiveness and safety. In Table 1, the main cells for each category of ATMPs are summarized.

Among adult stem cells, MSCs also known as mesenchymal stromal cells or medicinal stem cells, are multipotent stem cells that can differentiate into various cell types. They are primarily found in the bone marrow but can also be isolated from other tissues. MSCs have garnered significant attention for their therapeutic potential, particularly in regenerative medicine and tissue engineering. They are being investigated in over 1000 clinical trials for various applications, including treating of inflammatory diseases, tissue repair, and immune modulation. Their ability to respond to inflammation and promote tissue regeneration makes them a focal point in current biomedical research. Despite the promising potential of MSCs, challenges remain regarding their mechanisms of action, optimal isolation methods, and the intricacies of their differentiation pathways. Ongoing research aims to unlock their full therapeutic potential through an improved understanding of their biology and the development of effective clinical applications[8,9].

Table 1 Type of somatic cells employed in specific advanced therapy medicinal products (ATMPs)

ATMP	Intended use	Cells	Applications	Action in the tissue
GTMPs	The focus is primarily on the genetic material that is delivered into the patient's cells to correct or replace defective genes	Terminally differentiated: T-cells	Often modified for cancer immunotherapy (*e.g.*, CAR-T cells)	They can be performed *in vivo* (directly in the patient) or *ex vivo* (cells are modified outside the body and then reintroduced), which are leading to therapeutic, prophylactic, or diagnostic effect
		Hematopoietic stem cells	Used for genetic disorders affecting blood cells	
SCTMPs	Can involve cells that have been manipulated to alter their biological characteristics for therapeutic purposes, not intended to be used for the same essential function(s) in the recipient as in the donor	Terminally differentiated: Like fibroblasts and chondrocytes	Which are used for repairing specific tissues	They can repair or replace damaged tissues or to treat diseases, which are leading to therapeutic, prophylactic, or diagnostic effect. Unlike GTMPs, somatic cell therapy does not necessarily involve genetic modification but rather the application of cells to restore function
		Mesenchymal stem cells: Isolated adult tissues	These cells are known for their ability to differentiate into various cell types and are used in regenerative medicine	
		Induced pluripotent stem cells: These are reprogrammed adult cells	Can differentiate into any cell type, providing a versatile option for therapy	
TEPs	Cells that are used in combination with scaffolds to create functional tissues, not intended to be used for the same essential function(s) in the recipient as in the donor	Progenitor: Such as those derived from stem cells	Can differentiate into specific tissue types	They are designed to repair, regenerate, or replace damaged tissues or organs
		Engineered: Cells that have undergone substantial manipulation to achieve desired characteristics	For tissue repair or regeneration	

ATMP: Advanced therapy medicinal product; GTMP: Gene therapy medicinal product; SCTMP: Somatic cell therapy medicinal product; TEP: Tissue-engineered product; CAR-T: Chimeric antigen receptor T.

Induced pluripotent stem cells (iPSCs) are a type of pluripotent stem cell that can be generated directly by reprogramming adult somatic cells back into an embryonic-like pluripotent state through the forced expression of specific genes and factors important for maintaining the properties of ESCs. iPSCs are similar to ESCs in many aspects, including the expression of ESC markers, chromatin methylation patterns, ability to form embryoid bodies and teratomas, and potential to differentiate into various cell types. The breakthrough discovery of iPSCs allows researchers to obtain pluripotent stem cells without using embryos, providing a novel method to "de-differentiate" cells whose developmental fates were traditionally assumed to be determined. Patient-specific iPSCs carrying disease-relevant genetic backgrounds can be used to study disease mechanisms, evaluate drug activity and toxicity, and develop next-generation cell therapies. Tissues derived from iPSCs will be a nearly identical match to the cell donor, an important factor in disease modeling and regenerative medicine applications. Besides their advantages, challenges remain in ensuring the safety and efficacy of iPSC-based therapies, such as the potential for genetic and epigenetic abnormalities during reprogramming and differentiation[10].

Using stem cells for ATMPs presents several challenges that can impact their development, safety, and efficacy. Key challenges include: (1) Safety and efficacy concerns; (2) Regulatory inconsistencies; (3) Manufacturing challenges; and (4) Public perception and misuse[4].

SAFETY AND EFFICACY CONCERNS

Measurement plays a crucial role in developing therapeutic products and mimetic models as alternatives to animal testing. Accurate and precise measurement techniques are essential to assess the efficacy, safety, and quality of TEPs and models. However, challenges in measurement can jeopardize the advancement of therapeutic products and mimetic models in several ways, as depicted in Figure 2[11-15].

Metrology, the science of measurement, is increasingly recognized as pivotal in life sciences, particularly in the development of ATMPs. As discussed by Plant *et al*[16], the application of metrological principles involves meticulous planning and thorough documentation to diminish uncertainties and biases, thereby enhancing the reliability of experimental results in varied research contexts. Investigating the technical aspects of metrology in greater depth by focusing on precision and specificity is crucial for addressing the inherent variability present in biomedical research[17]. The importance of metrology in biological research has been highlighted[18], as it plays a crucial role in creating reference materials and defining measurement uncertainty. Due to the inherent variability of this field, standardization is essential for advancing biological research. Establishing precise, reliable and transparent measurement techniques, calibration, standards, and quality control is critical for achieving robustness and reproducibility in scientific research, particularly in developing therapeutic products. The Bureau International des Poids et Mesures (BIPM) is instrumental in this context.

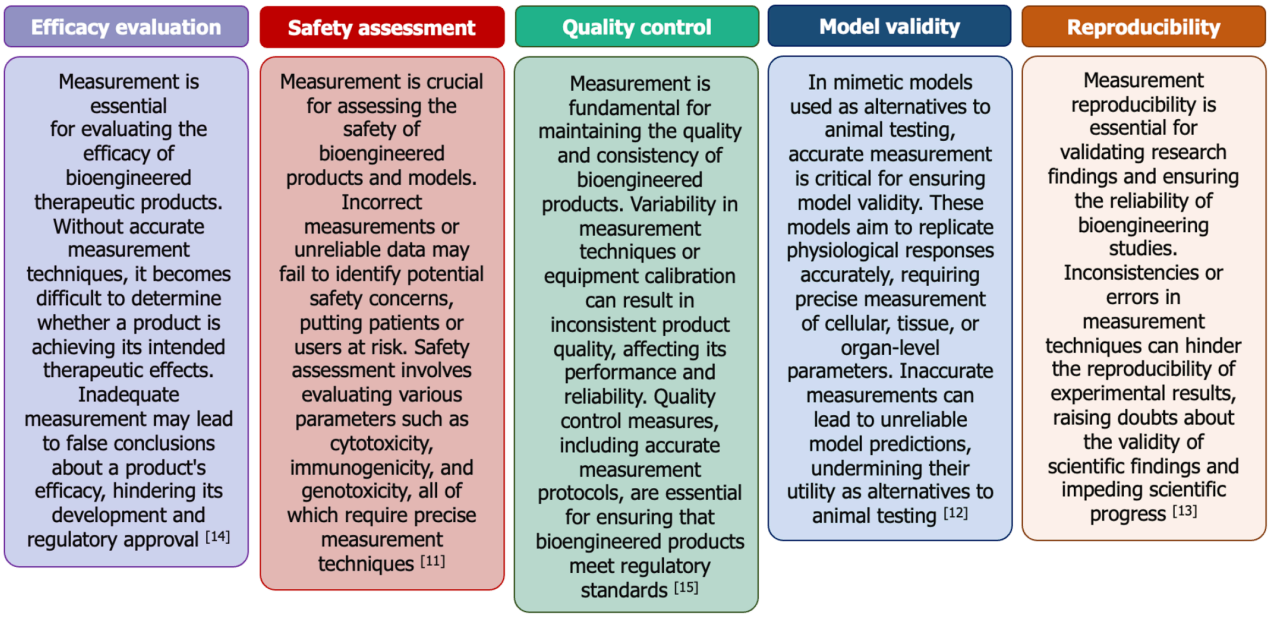

Figure 2 Measurement challenges that can hinder the progress of advanced therapeutic product development.

BIPM strives to improve measurement standards and methodologies in cellular analysis and product quality assurance. Collaborations with organizations like the European Association of National Metrology Institutes and initiatives like Quality by Design reflect the commitment of BIPM to enhance measurement accuracy and reliability, which is essential for the efficacy and safety of ATMPs applications and therapeutic interventions[19]. The use of MSCs and iPSCs in ATMPs raises important safety and efficacy concerns that need to be addressed.

In terms of safety concerns, we need to consider for MSCs: (1) Tumorigenicity (there is a risk of tumor formation associated with MSC therapies, particularly due to their ability to proliferate and differentiate and is heightened in cases where MSCs are derived from pluripotent sources or manipulated extensively, which may lead to uncontrolled growth *in vivo*; (2) Thromboembolic events (MSCs can express tissue factor - TF/CD142, which is procoagulant and can trigger coagulation cascades, linking to thromboembolic complications during the infusion of MSC products, necessitating careful monitoring and potential use of anticoagulants in clinical protocols[20]; (3) Immunogenicity (although MSCs are generally considered to have low immunogenicity, there is still a possibility of immune reactions, especially when using allogeneic/donor-derived cells); (4) Heterogeneity (MSCs are a heterogeneous population and inter-donor variations in their characteristics can impact their safety and efficacy as well as differences in isolation methods, culture conditions, and donor sources can lead to inconsistencies in product quality, which poses challenges for regulatory approval and clinical application[12]; (5) Quality control issues (variability in manufacturing processes can affect cell potency and safety, making it essential to establish stringent quality control measures throughout the production and handling of MSCs for ATMPs)[8].

In terms of safety concerns, we need to consider for iPSCs: (1) Genetic and epigenetic abnormalities (the reprogramming process to generate them can introduce genetic mutations and epigenetic aberrations that may compromise the safety and functionality of the cells); (2) Teratoma formation (the pluripotency and proliferative capacity of iPSCs and their derivatives increases the risk of uncontrolled growth and teratoma formation upon transplantation); and (3) Immunogenicity (even autologous iPSC-derived cells may trigger immune responses and rejection upon transplantation due to genetic and epigenetic changes acquired during reprogramming and culture). To address these concerns, researchers are exploring non-integrative methods for iPSC generation, such as episomal vectors and mRNA transfection, which offer higher safety while maintaining reprogramming efficiency. Genome editing tools like clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 are being used to correct genetic defects in patient-derived iPSCs before differentiation and transplantation. Ongoing research aims to further optimize the differentiation protocols, enhance the safety and efficacy of iPSC-derived cells, and develop robust quality control measures to ensure these cells' safe and effective use of these cells in ATMP applications[10].

In terms of efficacy concerns, we need to consider for MSCs: (1) Variability in cell characteristics (since MSCs are a heterogeneous population, and their properties can vary significantly based on the source (*e.g.*, bone marrow, adipose tissue, umbilical cord) and the individual donor and this variability can lead to inconsistent therapeutic outcomes, making it challenging to predict the efficacy of MSC-based therapies across different patients and conditions; (2) Differentiation potential (the ability of MSCs to differentiate into specific cell types is crucial for their therapeutic effectiveness; however, factors such as donor age, health status, and culture conditions can affect their differentiation capacity, leading to the limited or inconsistent differentiation that can result in suboptimal therapeutic effects, particularly in regenerative applications); (3) Quality control issues (inadequate quality assurance during manufacturing can lead to variations in cell potency and viability, ultimately affecting the efficacy of the final product; the lack of standardized protocols further complicates efforts to ensure consistent quality); (4) Immunogenicity and rejection (although MSCs are generally considered immunoprivileged, there is still a risk of immune responses, especially with allogeneic MSCs, and variability

in the immunogenic profile can lead to reduced efficacy or failure of the treatment due to immune rejection); (5) Long-term efficacy (many studies focus on short-term outcomes, and there is limited data on the long-term efficacy of MSC therapies and understanding how MSCs behave and maintain their effects in the long term is crucial for evaluating their overall therapeutic potential); and (6) Transport and handling (MSCs are living cells that require specific conditions to maintain their viability and functionality during transport and storage; any deviations from optimal conditions can compromise the efficacy of the product before administration, leading to questions about the reliability of the treatment) [21-23].

In terms of efficacy concerns, we need to consider for iPSCs: (1) Variability in differentiation efficiency (the differentiation efficiency of iPSCs can vary significantly between cell lines and may be lower compared to ESCs for certain lineages; (2) Epigenetic memory (iPSCs may retain an epigenetic memory of their somatic cell of origin, which can restrict their differentiation potential and skew their lineage commitment); and (3) Integration of transgenes (the use of integrative methods for iPSC generation, such as retroviral vectors, can disrupt tumor suppressor genes and increase the risk of tumorigenicity)[24].

REGULATORY CONSIDERATIONS

Bringing ATMPs to market involves navigating complex regulatory frameworks to ensure safety and efficacy. Challenges include defining appropriate regulatory pathways, addressing unique characteristics of ATMPs (such as the personalized nature and novel mechanisms), and establishing robust manufacturing processes[25,26].

Regulatory bodies play a crucial role in safeguarding public health by establishing and enforcing standards for new therapies. They assess preclinical and clinical data, oversee manufacturing practices, and conduct inspections to ensure compliance with regulations. Through rigorous evaluation, these bodies mitigate risks and promote the development of safe and effective treatments. However, the regulatory approach varies among agencies worldwide[27-29] (Figure 3). Each country's regulatory body has established comprehensive guidelines and regulations to manage the complex challenges presented by ATPs. They focus on robust scientific evaluation to ensure the safe integration of these innovative products into healthcare systems to provide advanced treatment options for patients.

Regulatory guidelines require ATMP manufactures to conduct cell differentiation assays[30], quantify impurities and metabolites, detect mycoplasma[31], perform sterility tests, analyze endotoxins, and use specific methods for quantifying dimethyl sulfoxide, penicillin, and streptomycin. The accuracy of these methodologies is essential to ensure the safety and efficacy of ATMPs[32,33]. This list is not exhaustive, and it is crucial to consult the specific guidelines of each country to identify the regulatory tests required for registering each type of ATMP. Each regulatory body also defines strict procedures for clinical trials to test ATMPs[34].

In terms of regulatory concerns are significant and multifaceted and we need to consider for both MSCs and iPSCs: (1) Compliance with good manufacturing practice (GMP) principles (stem cells must be produced under strict GMP conditions to ensure their safety, quality, and efficacy, including comprehensive documentation, quality control, and validation of manufacturing processes; compliance can be challenging, especially for academic institutions with limited experience in regulatory protocols; also, upgrading existing manufacturing processes to meet GMP standards can be costly and complex, particularly for large-scale production necessary for clinical trials; this often requires collaboration with industrial partners to achieve the necessary scale and compliance)[25]; (2) Inter-donor variability (can vary significantly between donors, leading to inconsistencies in product quality and therapeutic outcomes; establishing standardized protocols for cell isolation, expansion, and characterization is crucial but challenging); (3) Manufacturing standardization (there is a need for standardized procedures across different sources of MSCs to ensure consistent product quality and variability in manufacturing processes can complicate regulatory approval and clinical application); (4) Regulatory framework differences (varies significantly between regions and cells could be authorized under a hospital exemption clause, for example, in European Union); (4) Risk-based controls (the production of stem cells involves inherent risks, necessitating rigorous risk assessment and control measures throughout the manufacturing process, including ensure the safety, identity, purity, and potency of the final product); and (5) Quality consistency validation (ATMPs must undergo validation for quality consistency and successful demonstration of manufacturing processes; investigational ATMPs may not require full verification of analytical procedures, but authorized products must meet stringent validation standards). Addressing these regulatory concerns is essential for successfully developing and commercializing of stem cell-based ATMPs. In summary, Table 2 shows degree of cell manipulation, regulatory considerations, clinical trial design, and surgical considerations[8,35,36].

We focused on nine main regulatory frameworks. The United States Food and Drug Administration oversees ATMPs through various frameworks, including the Center for Biologics Evaluation and Research for biological products and the Center for Drug Evaluation and Research for drugs. Regulatory pathways such as investigational new drug applications, biologics license applications, and device premarket approvals are utilized[27].

ATMPs in the European Union fall under the European Medicines Agency Regulation 1394/2007 (Advanced Therapy Medicinal Products Regulation). This regulation encompasses gene cell therapy medicine, SCTM, and TEP. The European Medicines Agency provides centralized marketing authorization to ensure efficacy, safety, and compliance with quality standards[27]. Health Canada adopts a flexible regulatory framework tailored to the unique characteristics of ATMPs. Regulatory requirements prioritize safety and efficacy while fostering innovation in ATP development. The Medicines and Healthcare Products Regulatory Agency in the United Kingdom implements its regulations for ATMPs, focusing on stringent assessment and inspection processes to uphold patient safety and product effectiveness[37].

Table 2 Comparison between many aspects surrounding stem cells applied in advanced therapy medicinal products (ATMPs)

ATMPs	Degree of cell manipulation	Regulatory considerations	Clinical trial design	Surgical considerations
SCTMPs	Substantial manipulation	Often involves rigorous scrutiny of the manipulation processes and the resultant biological characteristics of the cells. Regulatory bodies require extensive data on safety and efficacy, particularly because these therapies may involve significant changes to the cells' original functions. Clinical trials must demonstrate not only the safety of the therapy but also its therapeutic benefits in the intended patient population	Often include endpoints that assess both the manufacturing process and the therapeutic outcomes. This may involve feasibility studies to ensure that the cells can be successfully harvested, manipulated, and reintroduced to the patient. The complexity of these therapies necessitates close coordination between clinical teams and manufacturing facilities	The administration may require less invasive procedures, depending on the therapy. For instance, T-cell therapies can often be administered through infusion after manipulation outside the body
TEPs	Substantial manipulation	The focus is more on the engineering processes and the ability of the product to integrate and function in the body. The regulatory framework may emphasize the physical and biological properties of the engineered tissues, requiring evidence that they can effectively repair or replace damaged tissues	May be more focused on demonstrating the functional integration of the engineered tissues and their ability to restore tissue function. The design of these trials often involves assessing the physical and biological properties of the implanted tissues and their long-term performance in the body	Typically involves more complex surgical procedures for implantation, which can introduce additional risks associated with surgery, such as infection or complications from the surgical site. The success of these products is closely tied to the surgical technique and the patient's ability to heal and integrate the new tissue

ATMP: Advanced therapy medicinal product; SCTMP: Somatic cell therapy medicinal product; TEP: Tissue-engineered product.

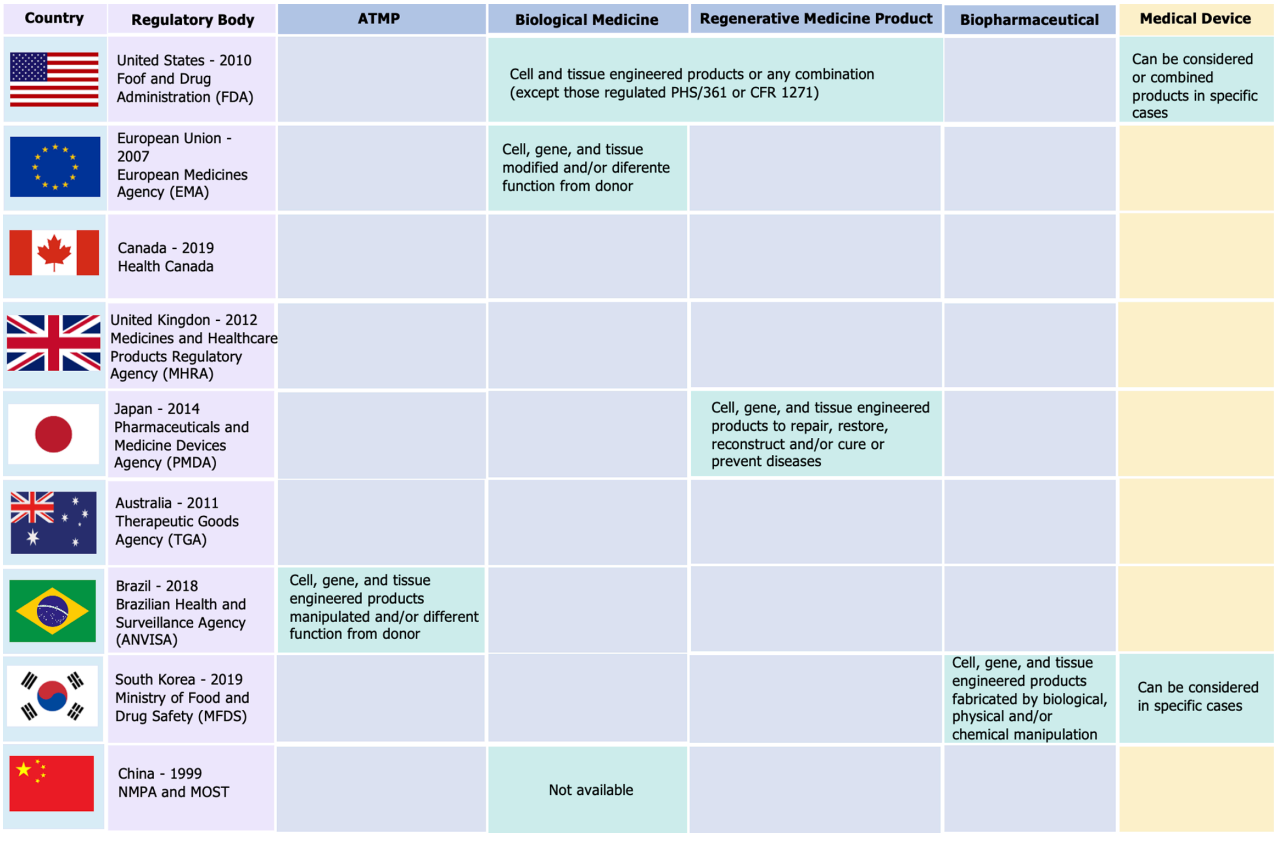

Figure 3 Regulatory landscape for advanced therapeutic products worldwide. ATMP: Advanced therapeutic product; MOST: National Health Commission of the People's Republic of China; NMPA: State Administration for Market Regulation.

Japan's Pharmaceuticals and Medicine Devices Agency has ensured adherence to quality, safety, and efficacy standards before market approval[38]. The Therapeutic Goods Administration in Australia regulates ATMPs under the Therapeutic Goods Act 1989 and relevant regulations, assessing them for safety, quality, and efficacy throughout their lifecycle, including post-market surveillance. The Brazilian Health and Surveillance Agency in Brazil has established specific regulatory frameworks for ATMPs, including Instruction Normative 270/2023, RDC 506/2021, and RDC 505/2021. These regulations govern good manufacturing practices, clinical trials, and product registration while ensuring compliance with rigorous standards for quality and safety[5]. The Ministry of Food and Drug Safety in South Korea is the

central regulatory authority. It has established a comprehensive regulatory framework to oversee the development, clinical testing, and commercialization of ATMPs. South Korea aims to harmonize its regulations with international standards[39].

The State Administration for Market Regulation and National Health Commission of the People's Republic of China regulates ATMPs as innovative biologics under the same framework as other pharmaceutical products, encompassing laws, regulations, departmental rules, and technical guidelines. China has seen a surge in policies promoting drug innovation, with the Center for Drug Evaluation significantly enhancing its capacity and efficiency in evaluating ATMPs. The ATMP industry in China is expanding rapidly. Although it lags in innovative target and indication coverage, there has been growth in diversity of product types, targets, and indications in recent years. This regulatory system encourages risk-based regulation and cross-discipline collaborations to advance more ATMPs towards market authorization in China, emphasizing expedited regulatory programs for efficient review processes, especially for highly innovative products from small companies. The National Drug Regulation Science Program of China has initiated the issuance of ATMP regulatory guidelines, supporting high-quality regulation of stem cell and gene therapies to achieve more targets in the coming years. Strengthening the regulatory framework involves updating guidelines, communicating effectively with stakeholders, and fostering partnerships with international regulatory agencies for convergence[40,41].

ETHICAL CONSIDERATIONS

Stem cell research brings critical ethical and societal issues to the forefront. Ethical concerns are primarily focused on the use of human embryos in research. The research ultimately involves their destruction, which raises questions about the embryo's moral status. This concern extends to issues of consent for the donation of tissues, particularly when it comes to reproductive cells or embryos that could potentially develop into a person[42]. Despite these challenges there is a growing societal consensus that the potential benefits of stem cell research may outweigh the ethical costs due to the promise of treating or even curing debilitating diseases[43]. In 2021, the International Society of Stem Cells Research released the "Guidelines for Stem Cell Research and Clinical Translation". The most crucial topics related to ethical and social considerations of stem cell use include: (1) Genetic material and confidential personal information; (2) Informed consent; (3) Genetic manipulation of the cells; and (4) Intellectual property and patents[44].

Moreover, the evolution of this field has led to a reduction in animal testing, aligning with societal values prioritizing compassion towards animals and ethical research practices[45] as reinforced by the Food and Drug Administration Modernization Act 2.0 which "allows for alternatives to animal testing for purposes of drug and biological product applications". Adhering to the principles of the "Replacement, Reduction, and Refinement" in animal research, stem cell studies contribute to more ethical scientific protocols and heighten the integrity and public perception of scientific research[46,47].

For instance, 3D bioprinting has been used to create complex tissue models that closely mimic human physiology, such as liver and cardiac tissues, allowing for precise replication of the human disease environment and drug responses[48]. High-throughput methods for creating multicellular spheroids, which more accurately represent the *in vivo* tumor microenvironment, have been developed to enhance cancer research without relying on animal models[49]. Human liver spheroids and advanced 3D bioprinting techniques are emerging as effective alternatives to animal testing for evaluating hepatotoxicity and drug efficacy in treating liver diseases, such as non-alcoholic steatohepatitis[50]. These practices foster trust in scientific research and stimulate the development of innovative methods that are both humane and potentially more indicative of human biological responses[51,52].

TEP

Recent advancements include in TE the integration of biomaterials, cellular components, and engineering principles to fabricate functional tissues and organs[13,53,54] (Figure 4). This multidisciplinary approach has revolutionized regenerative medicine, offering novel tissue repair and organ replacement solutions. Recent breakthroughs in TE have revolutionized the field, with significant advancements in CRISPR technology, bioinformatics, and nanotechnology[55]. 3D bioprinting, organ-on-a-chip, and stem cell technologies have seen remarkable progress[52,56]. Nanoengineering has significantly enhanced the performance and functionalities of biomaterials with potential applications in developing biomedical treatments and techniques[57]. Further developments involve the utilization of immunoengineering and regenerative immunotherapies to guide tissue reconstruction[58]. Notably, incorporating techniques like electrical stimulation and nanoparticle synthesis to promote cell proliferation and differentiation have emerged[59]. These breakthroughs collectively represent the cutting edge of TE, which can potentially transform various industries and improve human health. Stem cell technology emerged as a disruptive force in TE by challenging conventional paradigms and offering unprecedented therapeutic potential. The 3D stem cell culture systems in TEPs offers numerous benefits over traditional 2D cultures. Here are the key advantages: Enhanced physiological relevance: (1) Mimicking *in vivo* conditions: 3D cell cultures better replicate the tissue's natural architecture and microenvironment of tissues compared than 2D cultures. This allows for more accurate modeling of cellular behavior, interactions, and responses to stimuli, which is crucial for studying tissue development and disease processes[60]; and (2) Improved cell-cell and cell-extracellular matrix (ECM) interactions: In 3D cultures, cells can interact with each other and with the ECM in a manner that closely resembles their behavior *in vivo*. This promotes more natural cell proliferation, differentiation, and function, leading to more relevant results in tissue engineering applications[60]. Scalability and versatility: (1) Scalable production: 3D culture systems can be designed to produce large quantities of tissue constructs, making them suitable for various applications in regenerative medicine and tissue engineering. This scalability is essential for developing clinically relevant

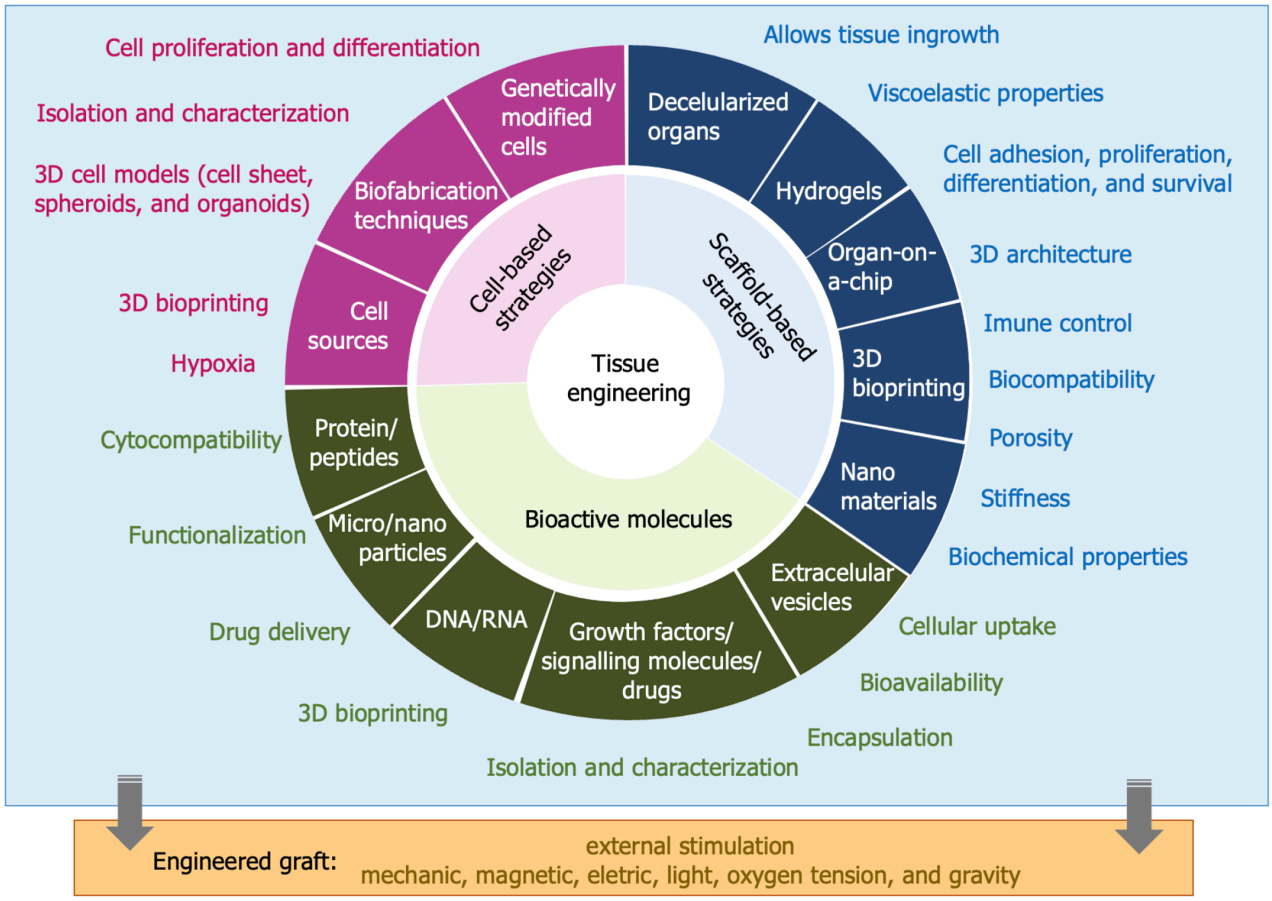

Figure 4 Tissue engineering basics. Three essential components required for tissue regeneration are: (1) Living cells that can proliferate and differentiate to form new tissue; (2) Scaffolds that provide structural support for cell attachment and tissue formation; and (3) Bioactive molecules that promote cell proliferation, differentiation, and tissue development. 3D: Three-dimensional.

products[61]; and (2) Diverse applications: 3D cell cultures can be applied for various 3D microenvironments, such as in many scaffolds (hydrogels, decellularized matrix) and microfluidic systems. Enhanced biocompatibility and integration: (1) Better biocompatibility: 3D cell cultures often utilize biomaterials that mimic the natural ECM, improving cell adhesion and promoting tissue integration when implanted *in vivo*. This is critical for the success of tissue-engineered products[61]; and (2) Facilitated nutrient exchange: Unlike 2D cultures, where nutrient access is uniform, 3D cultures create gradients of nutrients and oxygen, which can influence cell behavior and viability. This feature is vital for maintaining the health and functionality of engineered tissues over time[60].

Despite the advantages, several challenges remain in 3D stem cell culture: (1) Standardization (there is a lack of standardized protocols for 3D culture systems, leading to variability in results and complicating comparisons across studies); (2) Cost and complexity (the materials and technologies required for 3D cultures can be expensive, and the complexity of these systems may require specialized expertise for effective management and analysis); (3) Assessment and analysis (current assays for analyzing 3D cultures are less developed compared to those for 2D cultures, making it difficult to quantify outcomes and assess cellular responses consistently.

Several emerging sources of MSCs are being explored for their potential use in TEPs: (1) Menstrual blood-derived MSCs (can be obtained non-invasively from healthy women; have shown good proliferative capacity, multi-lineage differentiation potential, and immunomodulatory properties; however, challenges remain in standardizing the manufacturing process due to potential variability based on the day of the menstrual cycle when the cells are obtained); (2) Dental pulp-derived MSCs (obtained from extracted teeth; exhibit characteristics similar to bone marrow-derived MSCs and have been explored for regenerative therapies in dentistry and orthopedics; while preclinical and early clinical studies are promising, more translational research is needed to consolidate the results and establish standardized manufacturing protocols for ATMP development); and (3) iPSC-derived MSCs (can be reprogrammed to generate MSCs, providing an unlimited and consistent cell source for ATMP manufacturing; have shown comparable characteristics to MSCs from other sources and may offer advantages in terms of scalability, consistency, and potential for genetic modification; however, challenges remain in ensuring the complete elimination of residual undifferentiated iPSCs in the final product and establishing robust quality control measures to mitigate the risk of tumorigenicity)[62].

While specific numbers of approved TEPs can vary, it is noted that TEPs constitute less than 5% of all ATMPs currently in clinical trials. The approval landscape is evolving, with ongoing clinical trials and regulatory adaptations to facilitate introducing more TEPs into the market. Currently, the total number of TEPs that have received regulatory approval is limited, highlighting the challenges associated with their development and commercialization in regenerative medicine

and tissue engineering[63].

Until now, the number of approved TEPs remains relatively low compared to the overall number of ATMPs. As of recent reports, the first TEP to receive approval was ChondroCelect, an autologous cartilage cell-based product in association with collagen membrane for patellar and trochlear articular cartilage repair approved by European Medicines Agency in 2009, however was withdrawn in 2016. Since then, the development of TEPs has been slow, with only a few products reaching the market: (1) MACI (Genzyme): Matrix-applied characterized autologous cultured chondrocytes for repair of symptomatic cartilage defects of the knee in combination with porcine collagen scaffold and a first combined ATMP approved by European Medicines Agency in 2013 and Food and Drug Administration in 2016, however the authorization has expired; (2) Holoclar (considered an orfan medicine): *Ex vivo* expanded autologous human corneal epithelial cells (limbic biopsy) containing stem cells on a fibrin membrane (cell sheet) for causes of physical or chemical ocular burns, causing limbal stem cell deficiency, approved by European Medicines Agency in 2015; and (3) Spherox (CO.DON): Spheroids (10-70 spheroids/cm^2 suspension for implantation) of human autologous matrix-associated chondrocytes for repair of symptomatic articular cartilage defects of the femoral condyle and the patella of the knee, approved by European Medicines Agency in 2017[63].

CONCLUSION

ATMPs are at the forefront of medical innovation, offering new hope for patients with previously untreatable conditions. As the field continues to evolve, the focus remains on ensuring safety, efficacy, and accessibility while navigating the complexities of regulatory frameworks, market dynamics, and pricing and reimbursement[64-66]. The ongoing development and implementation of ATMPs could revolutionize treatment paradigms across various medical disciplines, significantly impacting patient care and outcomes.

Required safety-related changes can inadvertently reduce the safety or efficacy of the ATMP. For example, modifications such as removing serum or feeder layers in culture can significantly decrease the yield of desired stem cells, necessitating alternative approaches to ensure safety and efficacy. There is a critical need to formally demonstrate the efficacy of stem cell therapies, as invasive procedures are often involved. The challenge lies in providing robust clinical evidence that meets regulatory standards, especially in life-threatening diseases where traditional therapies may not be effective[67].

There are inconsistencies between regulatory authorities regarding the advice and requirements for stem cell-based ATMPs. This can complicate the process of preserving the drug's potency during manufacturing scale-up and validation, leading to potential product rejection. Also, regulatory bodies often require changes to clinical protocols for generating regulatory-grade data without fully understanding the biological mechanisms involved. This can result in protocols that are not suitable for specific treatments, complicating the development process[67].

The production of stem cell-based ATMPs involves complex and variable processes that must comply with GMP standards. Ensuring consistency and reproducibility in the manufacturing process is essential but can be difficult due to the inherent variability of biological materials. Maintaining high-quality standards throughout the manufacturing process is critical. This includes ensuring that the final product is safe and effective, which can be complicated by the unique characteristics of stem cells.

The proliferation of unregulated stem cell therapies can lead to public skepticism and fear regarding legitimate stem cell treatments. Patients may be exposed to ineffective or harmful therapies, undermining trust in scientifically validated treatments. The subjective nature of assessing outcomes in some stem cell therapies can lead to inflated reports of efficacy due to placebo effects, complicating the evaluation of true therapeutic benefits[4,67]. Addressing these challenges requires a collaborative approach involving researchers, regulators, and healthcare providers to ensure that stem cell-based ATMPs are developed safely and effectively, with a strong emphasis on education and public awareness.

Emerging trends

3D bioprinting: Advances in bioprinting technology enable the creation of complex tissue-like structures with precise spatial organization of cells and hydrogels, enhancing the potential for functional tissue engineering[68]; and dynamic culture systems: Innovations in perfusion (microfluidics) and bioreactor systems provide dynamic environments that mimic physiological conditions, improving nutrient and oxygen delivery to 3D cultures and supporting cell viability and function[69].

FOOTNOTES

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ORIGINAL ARTICLE

Basic Study

Gamma-aminobutyric acid enhances miR-21-5p loading into adipose-derived stem cell extracellular vesicles to alleviate myocardial ischemia-reperfusion injury *via* TXNIP regulation

Feng-Dan Wang, Yi Ding, Jian-Hong Zhou, En Zhou, Tian-Tian Zhang, Yu-Qi Fan, Qing He, Zong-Qi Zhang, Cheng-Yu Mao, Jun-Feng Zhang, Jing Zhou

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Abstract

BACKGROUND

Myocardial ischemia-reperfusion injury (MIRI) poses a prevalent challenge in current reperfusion therapies, with an absence of efficacious interventions to address the underlying causes.

AIM

To investigate whether the extracellular vesicles (EVs) secreted by adipose mesenchymal stem cells (ADSCs) derived from subcutaneous inguinal adipose tissue (IAT) under γ-aminobutyric acid (GABA) induction (GABA-EVs^{IAT}) demonstrate a more pronounced inhibitory effect on mitochondrial oxidative stress and elucidate the underlying mechanisms.

METHODS

We investigated the potential protective effects of EVs derived from mouse ADSCs pretreated with GABA. We assessed cardiomyocyte injury using terminal deoxynucleotidyl transferase dUTP nick end-labeling and Annexin V/propidium iodide assays. The integrity of cardiomyocyte mitochondria morphology was assessed using electron microscopy across various intervention backgrounds. To explore the functional RNA diversity between EVs^{IAT} and GABA-EVs^{IAT}, we em-

ployed microRNA (miR) sequencing. Through a dual-luciferase reporter assay, we confirmed the molecular mechanism by which EVs mediate thioredoxin-interacting protein (TXNIP). Western blotting and immunofluorescence were conducted to determine how TXNIP is involved in mediation of oxidative stress and mitochondrial dysfunction.

RESULTS

Our study demonstrates that, under the influence of GABA, ADSCs exhibit an increased capacity to encapsulate a higher abundance of miR-21-5p within EVs. Consequently, this leads to a more pronounced inhibitory effect on mitochondrial oxidative stress compared to EVs from ADSCs without GABA intervention, ultimately resulting in myocardial protection. On a molecular mechanism level, EVs regulate the expression of TXNIP and mitigating excessive oxidative stress in mitochondria during MIRI process to rescue cardiomyocytes.

CONCLUSION

Administration of GABA leads to the specific loading of miR-21-5p into EVs by ADSCs, thereby regulating the expression of TXNIP. The EVs derived from ADSCs treated with GABA effectively ameliorates mitochondrial oxidative stress and mitigates cardiomyocytes damage in the pathological process of MIRI.

Key Words: Extracellular vesicles; Myocardial ischemia-reperfusion injury; Adipose-derived mesenchymal stem cells; Gamma-aminobutyric acid; Thioredoxin-interacting protein

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Core Tip: Extracellular vesicles secreted by adipose mesenchymal stem cells derived from subcutaneous inguinal adipose tissue under γ-aminobutyric acid induction demonstrate a pronounced inhibitory effect on mitochondrial oxidative stress and showcases a safeguarding impact on the cardiomyocytes. The protective effects may result from extracellular vesicle microRNA-21-5p targeting thioredoxin (TXNIP)-interacting protein, regulating TXNIP-interacting protein-TXNIP complex formation and subsequent enhancing the antioxidant activity of TXNIP.

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INTRODUCTION

Myocardial infarction (MI) is a grave cardiovascular ailment triggered by coronary occlusion, resulting in acute and persistent ischemia and hypoxia within the myocardium[1]. However, upon reestablishing blood flow to the previously occluded vessels, a phenomenon known as myocardial ischemia-reperfusion injury (MIRI) may occur, leading to potential secondary damage of the perfused myocardium, which continues to pose a significant challenge in clinical practice[2]. During the phase of MIRI, mitochondrial oxidative stress emerges as the predominant form of cardiomyocyte demise[3]. Hence, notwithstanding the expeditious reopening of occluded blood vessels and subsequent restoration of blood flow, the initial mitochondrial oxidative stress will further evolve into passive programmed cell death or even irreversible necrosis[4].

This culminates in an onslaught of mitochondrial oxidative stress, wherein excessive generation of mitochondrial reactive oxygen species (mROS) triggers a cascade of detrimental events, potentially compromising mitochondrial function[5,6]. Furthermore, heightened levels of mROS and hypoxia induce an upregulation of thioredoxin-interacting protein (TXNIP)[7], which impairs the resilience of cardiomyocytes to hypoxic conditions and incites mitochondrial oxidative stress which ultimately culminates in the collapse of mitochondrial membrane potential and functional impairment or even destruction, resulting in various programmed cell death events within cardiomyocytes after reperfusion therapy administration[8,9]. TXNIP impedes the reductive capacity of thioredoxin (TRX), thereby obstructing its function in regulating oxidative stress at both the cellular and subcellular levels with finesse[10]. Henceforth, targeting TXNIP emerges as a promising therapeutic strategy for safeguarding against MIRI encompassing mitochondrial oxidative stress triggered by hypoxia-oxygenation recovery arising from the restoration of blood flow[11-13].

Accumulating evidence has demonstrated that subcutaneous inguinal adipose tissue (IAT), particularly adipose-derived mesenchymal stem cells (ADSCs), possess remarkable regulatory functions regarding adipose tissue, peripheral organs, systemic inflammation, endoplasmic reticulum stress, and mitochondrial stress[14-16]. Moreover, these effects can be further augmented under the influence of γ-aminobutyric acid (GABA)[17]. The aforementioned biological effects are primarily believed to be mediated by extracellular vesicles (EVs), necessitating meticulous examination. EVs facilitate intercellular communication by transferring proteins, mRNAs, and microRNAs (miRs) in a manner that can be broad or

specific to certain cell types. The molecular composition of EVs reflects that of their source cells and can influence the biological functions of target cells, tissues, and organs such as differentiation, proliferation, migration, secretion, and apoptosis[18,19]. Changes in the microenvironment and physiological state of EV-producing cells can also impact both the contents and biological function of EVs[20,21]. Nevertheless, uncertainty persists regarding the role of EVs in mediating the GABA-induced impacts of ADSCs from IAT on mitigating mitochondrial dysfunction and oxidative stress. Moreover, a comprehensive exploration into the underlying mechanisms responsible for these effects is still pending.

The objective of this study was to investigate whether EVs derived from GABA-induced ADSCs from IAT (GABA-EVs^{IAT}) exert a more pronounced impact on ameliorating MIRI than EVs derived from ADSCs from IAT (EVs^{IAT}) and elucidate its underlying mechanisms to offer a potential efficacious approach for enhancing MIRI.

MATERIALS AND METHODS

MIRI mouse model

The Institutional Ethics Committee of Shanghai Ninth People's Hospital (Shanghai, China) granted proper approval for the animal care and procedures. The animal experimental protocols were meticulously followed in strict accordance with Directive 2010/63/EU. Male C57BL/6 mice, aged 8 weeks, were obtained from Shanghai Jessie Experimental Animal Co., Ltd. (Shanghai, China), and the establishment of transgenic mouse models was conducted by Shanghai Southern Model Organisms Co., Ltd. (Shanghai, China). These mice were provided with unrestricted access to standard mouse chow and water while being housed under specific pathogen-free conditions (maintained at a temperature range of 20-24 °C with humidity levels between 50% and 60%). All invasive procedures were conducted under anesthesia. Anesthesia induction was achieved using an anesthesia box infused with 2% isoflurane (RWD Life Technology Co., Ltd., Shenzhen, China) for 3 min, followed by maintenance anesthesia administered through an animal anesthetic mask containing a concentration of 1% isoflurane. Carbon dioxide inhalation was utilized as a humane method for euthanizing the mice. The traditional Cre-loxP system was used to construct all gene knockout (KO) mice, based on relevant research[22]. Cyagen Biotechnology Co., Ltd. (Jiangsu, China) and Shanghai Model Organisms Center, Inc. (Shanghai, China) completed the construction of all gene KO mice in this study.

A total of 80 male C57BL/6 mice, weighing between 20 g and 24 g, were employed for the experimental trials. We followed the detailed protocol outlined by Gao *et al*[23] to create the MI model. This involved 30 min of left coronary artery (LCA) ligation. Blood flow occlusion was temporarily induced using a slipknot and subsequently released to simulate reperfusion and restore blood supply to the ischemic regions. Successful confirmation of MI induction was based on dynamic electrocardiograph changes characterized by ST-segment elevation. As part of our control group, sham-operated mice underwent an identical procedure with the exception that the knot on the LCA remained loosely tied.

Transthoracic echocardiography

The echocardiography was conducted on the 3rd day post MIRI surgery, utilizing the state-of-the-art Vevo 770 high-resolution imaging system (FUJIFILM, Japan) in small animal models, to meticulously evaluate (M mode) ejection fraction and fractional shortening across three consecutive cardiac cycles.

Evaluation of area at risk and infarct size

After 12 h of MIRI, the LCA was re-ligated at the same level using a knot. Subsequently, under 1.5%-2% isoflurane anesthesia administration, the chest wall was reopened to elegantly expose the heart. Following this, meticulous reconnection of the LCA took place while gently applying a clamp to the aortic arch. Meanwhile, reverse injection of 1% Evans blue (dissolved in normal saline) through the ascending aorta resulted in non-infarcted areas displaying an exquisite blue coloration. Subsequently, with utmost care and precision, the heart was removed and rinsed with normal saline before being horizontally sliced into approximately 1 mm thick sections below the ligation level. Each section underwent immediate incubation in phosphate-buffered saline containing 1.5% triphenyltetrazolium chloride at 37 °C for 20 min. Utilizing Image-Pro Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, United States) with finesse and expertise, both the infarct area and area at risk (AAR) zone were quantified accordingly. The calculation involved determining infarct size/AAR × 100% as well as AAR/left ventricle area (LV) × 100%, respectively.

Isolation and culture of mouse ADSCs and neonatal mouse cardiomyocytes

The ADSCs were obtained from the IAT of C57BL/6 mice using a previously established protocol[24]. Flow cytometry analysis was conducted to characterize the ADSCs (Supplementary Figure 1), with a focus on cluster of differentiation 34 (CD34), CD105 (as negative controls), and CD106 as well as CD29, CD45, and CD90 (as positive cell surface markers). Primary neonatal mouse cardiomyocytes (NMCMs) were extracted from 1-day-old neonatal C57BL/6 mice and the protocol has been previously described[25].

In vitro cardiomyocyte hypoxia model

The creation of an *in vitro* NMCMs hypoxia model involved subjecting cells to a meticulously controlled environment devoid of oxygen and low in glucose, using DMEM. This was achieved by maintaining 5% CO_2 and 95% N_2 for a duration of 2 h. Subsequently, the incubation conditions were switched to normoxic fetal bovine serum (FBS)-free medium for 12 h. After treatment, the myocardial cells were collected and subjected to thorough analysis.

GABA-induced ADSCs

ADSCs were seeded in complete medium (DMEM/F12 with 10% EV-free FBS [EXO-FBS-50A-1; System Biosciences, Palo Alto, CA, United States]) for 24 h. The ADSCs were further cultured in a GABA-rich environment at a concentration of 5 μM, and the supernatant was collected for EV extraction after 48 h.

Isolation and characterization of EVs

The EVs were isolated from cultured ADSCs using differential velocity centrifugation[24], and the concentration of EVs was quantified using the bicinchoninic acid assay (BCA). Briefly, the cell culture supernatant was centrifuged at 2000 × *g* for 30 min at 4 °C to eliminate any cellular debris. Subsequently, the resulting supernatant was collected and further centrifuged at 100000 × *g* for 70 min to precipitate the EVs. To eliminate any contaminating proteins, the supernatant was discarded and the EVs were resuspended in phosphate-buffered saline (PBS). The size distribution and concentration of the EVs were determined utilizing the NanoSight NS300 instrument (Malvern Instruments, Malvern, United Kingdom), and their morphology was examined by transmission electron microscopy (TEM) and Nanosight.

EV injections and confirmation of EV uptake by cardiomyocytes

The EVs were injected into the border zone of the infarcted heart at three specific sites, immediately following MI surgery, at a dosage of 1 μg/1 g mouse body weight and the administered volume of EVs at each injection site was one-third of the total volume to be injected. These EVs were labeled with PKH67 (Cat. MINI67; Sigma-Aldrich, St. Louis, MO, United States), while the NMCMs were stained with phalloidin (Cat. A12379s; Thermo Fisher Scientific, Waltham, MA, United States). Each culture of NMCMs was treated with 20 μg EV solution (determined by the BCA), stained with 5 mL PKH67, and incubated for 2 h to facilitate endocytosis by the NMCMs. After being washed three times with PBS, the cells were fixed in 4% paraformaldehyde for 20 min and DAPI stain was used to label the nucleus. An inverted microscope was employed to observe the engulfed EVs within the cardiomyocytes.

Terminal deoxynucleotidyl transferase dUTP nick end-labeling assay

Sections were fixed in 4% paraformaldehyde for 15 min and then treated with 1 μg/mL proteinase K for 10-20 min. Then the sections were briefly refixed in 4% paraformaldehyde and washed twice with PBS for 5 min each. A positive control was prepared and excess liquid was removed from the slides. Next, 100 μL equilibration buffer was added to each slide, and the slides were carefully covered with plastic coverslips and incubated for 5-10 min. The plastic coverslips and excess buffer were carefully removed from the slides. TdT reaction mix (100 μL) or negative control (NC) mix was added instead of TdT reaction mix as a NC. Then the slides were carefully covered again with plastic coverslips and incubated at 37 °C for 1 h. The sections were washed three times with PBST for 10 min each before the slides were transferred to a slide rack. The sections were washed twice with dH_2O for 3 min each, followed by dehydration once in ethanol solutions (50%, 70%, 95%) and twice in pure ethanol (100%), with each step lasting 3 min. Then the sections were incubated twice in xylene for 3 min each before mounting slides using DPX and applying micro cover glasses while ensuring no air bubbles were trapped during this process.

Dual-luciferase reporter assay

HEK 293T cells were meticulously cultured in 24-well plates until they were approximately 70% confluent. Subsequently, a co-transfection was performed employing miR mimics and PGL3 luciferase plasmids harboring either wild-type, NC, or mutated TXNIP 3′ untranslated region (3′UTR) sequences. Following a 12-h incubation period, the cells were transferred to 96-well luciferase assay plates. Thereafter, the Dual-GLOTM Luciferase Assay System (Cat. E2920; Promega, Madison, WI, United States) was employed to determine the ratio of firefly to Renilla luciferase activity.

Oxygen consumption measurements

The Seahorse XFe96 Extracellular Flux Analyzer, in combination with the Seahorse Cell Mito Stress Test Kit (Agilent Technologies, Wilmington, DE, United States), were utilized to measure the oxygen consumption rates (OCRs) of NMCMs. The cells were cultured on Seahorse cell culture plates and subjected to the established protocol[26]. Prior to measurement, the culture media were replaced with Seahorse XF DMEM supplemented with 5 μM glucose, 1 μM pyruvate, and 10 μM glutamine for 1 h. Following incubation at 37 °C in a CO_2-free incubator for 1 h, the OCRs were assessed under both basal conditions and in the presence of oligomycin (1.5 mM), FCCP (1 mM), and rotenone/antimycin A (0.5 mM). To normalize the results, Hoechst 33342 staining was employed to determine total cell number at the conclusion of the Seahorse experiment.

Quantitative polymerase chain reaction and western blotting

We utilized the RNAiso Plus extraction reagent (Cat. 9108; Takara, Dalian, China) to isolate EV-associated RNA. To generate microRNA (miRNA) cDNA, we employed stem-loop primers from Ribobio Biotech and amplified the resulting cDNA using the SYBR Green-based quantitative polymerase chain (qPCR) reaction method. U6 small nuclear RNA was used as an internal control for normalization purposes. RIPA was implemented to extract protein from cardiac tissues and cardiomyocytes while antibodies against tumor susceptibility 101 (TSG101), CD63, CD81, thioredoxin-interacting protein (TXNIP) were obtained from Abcam (Cambridge, MA, United States) with α-tubulin antibodies provided by Cell Signaling Technology (Danvers, MA, United States).

TEM

To perform TEM, heart tissues were promptly immersed in 2.5% glutaraldehyde solution at 4 °C for fixation. The fixed samples underwent three rinses with a buffer containing 0.1 mM cacodylate trihydrate and were subsequently post-fixed using a solution of 1% osmium tetroxide for a duration of 1 h. Following three additional washes with PB, the samples were dehydrated through ethanol gradients, treated with acetone, and ultimately embedded in ethoxyline resin. Subsequently, ultrathin sections were carefully placed onto a copper grid before capturing images utilizing the FEI electron microscope (Tecnai G2 Spirit 120 kV; FEI Italia, Vercelli, Italy).

Statistical analysis

Data analysis was conducted utilizing the SPSS 19.0 software package (IBM Corp., Armonk, NY, United States). The assessment of normal distribution for the data was performed employing the Shapiro-Wilk test. Categorical variables were subjected to analysis using either the Pearson's χ^2 test (for $n \geq 5$) or Fisher's exact test (for $n < 5$), followed by multiple comparisons with Bonferroni correction. Continuous variables were analyzed with one-way analysis of variance, and subsequent post-hoc multiple comparisons were performed using the Student-Newman-Keuls test. Nonparametric testing for multiple independent samples was carried out utilizing the Kruskal-Wallis test, and post hoc comparisons were made with the Dunn-Bonferroni test.

RESULTS

Characterization of ADSCs and ADSC-derived EVs

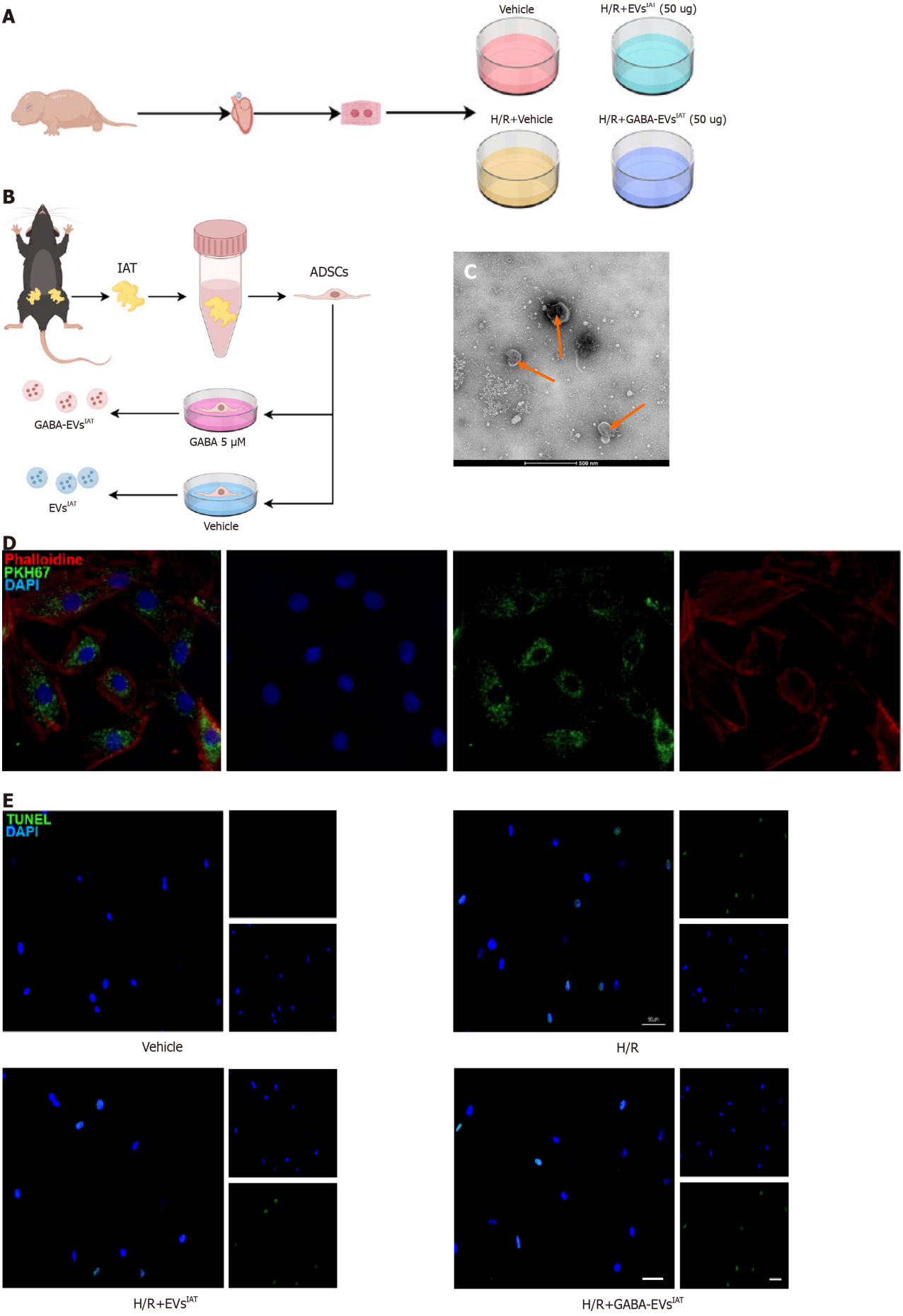
A schematic diagram depicting the identification of ADSCs and the extraction of their EVs is presented in Figure 1A and B. The successful isolation of ADSCs from mouse adipose tissue was confirmed using flow cytometry, which showed that the cell surface markers CD29, CD45, and CD90 were expressed positively (> 80%), while CD34, CD105, and CD106 had low/negative expression (Supplementary Figure 1A). Sequential centrifugation was employed to obtain ADSC-derived EVs (ADSC-EVs), which then were characterized using TEM and the Nanosight instrument (Figure 1C and Supplementary Figure 1B). These analyses revealed that the collected vesicles primarily consisted of EVs with an average size of 115 nm (range: 50-150 nm). The EVs markers CD63, CD81, and TSG101 were identified by Western blotting (Supplementary Figure 1C). The uptake of PKH26-labeled EVs by cultured NMCMs was verified by fluorescence microscopy (Figure 1D).

GABA-EVs^{IAT} reduce injury in NMCMs subjected to hypoxia and reoxygenation

To investigate the potential of GABA-EVs^{IAT} in preventing or mitigating hypoxia and reoxygenation (H/R)-induced injury in NMCMs, injury-related markers were assessed using AnnexinV/PI (G1511; Servicebio, Wuhan, China) assay and terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL) assay (G1501; Servicebio) in NMCMs exposed to H/R (6 h of hypoxia followed by 12 h of reoxygenation) and treated with EVs^{IAT} or GABA-EVs^{IAT} prior to H/R. As demonstrated by the TUNEL assay results (Figure 1E), exposure to EVs^{IAT} reduced the apoptotic rate of NMCMs (P = 0.0035 for H/R + EVs^{IAT} *vs* H/R group). However, the downregulation of apoptosis was significantly greater after treatment with GABA-EVs^{IAT} (P = 0.0083 for H/R + GABA-EVs^{IAT} *vs* H/R + EVs^{IAT} group). Similarly, significant differences were observed in GABA-EVs^{IAT}-treated cells using AnnexinV/PI assay (P = 0.0009 for H/R + GABA-EVs^{IAT} *vs* H/R + EVs^{IAT} group) (Figure 1F).

GABA-EVs^{IAT} mitigates NMCMs mitochondrial oxidative stress levels and enhances mitochondrial function during H/R

The early pathogenesis of MIRI involves excessive mitochondrial oxidative stress as a pivotal mechanism[27]. We tried to evaluate cardioprotective effects of GABA-EVs^{IAT} in improving mitochondrial function and alleviating excessive oxidative stress in NMCMs during H/R. The findings suggest that in the context of H/R (Figure 2A), EVs^{IAT} has the ability to enhance the mitochondrial membrane potential of NMCMs (P = 0.0231 for H/R + EVs^{IAT} *vs* H/R group). However, it is noteworthy that GABA-EVs^{IAT} exhibits a more pronounced efficacy (Figure 2B), primarily characterized by a greater normalization of the membrane potential compared to group H/R + EVs^{IAT} (P = 0.0225 for H/R + GABA-EVs^{IAT} *vs* H/R + EVs^{IAT} group). The findings from mitochondrial OCR also align with the aforementioned conclusions (Figure 2C). In the context of H/R, EVs^{IAT} exhibit the potential to enhance both basal and maximal oxygen consumption of NMCMs mitochondria, thereby partly stabilizing aerobic respiration function in mitochondria (basal OCR: P = 0.0077 for H/R + EVs^{IAT} *vs* H/R group; maximal OCR: P = 0.0267 for H/R + EVs^{IAT} *vs* H/R group). Conversely, GABA-EVs^{IAT} demonstrate a heightened efficacy in ameliorating mitochondrial aerobic respiration function under pathological conditions induced by H/R (basal OCR: P = 0.0081 for H/R + GABA-EVs^{IAT} *vs* H/R + EVs^{IAT} group; maximal OCR: P = 0.0006 for H/R + GABA-EVs^{IAT} *vs* H/R + EVs^{IAT} group). In addition, under H/R conditions, the activation level of lysosomes in NMCMs shows a moderate decrease regulated by GABA-EVs^{IAT} (Figure 2D). This suggests that GABA-EVs^{IAT} can reduce mitochondrial damage and subsequently diminish the process of lysosomal activation for clearing damaged mitochondria (P = 0.0083 for H/R + GABA-EVs^{IAT} *vs* H/R group). The morphological assessment revealed that under H/R conditions, EVs^{IAT} and GABA-EVs^{IAT} exhibited a remarkable ability to partially fortify the intricate architecture of myocardial cell mitochondria cristae as demonstrated by transmission electron microscope assay results (Figure 2E).


A
Vehicle
H/R+EVs^IAI (50 ug)
H/R+Vehicle
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B
IAT
ADSCs
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GABA 5 μM
EVs^IAT
Vehicle
C
500 nm
D
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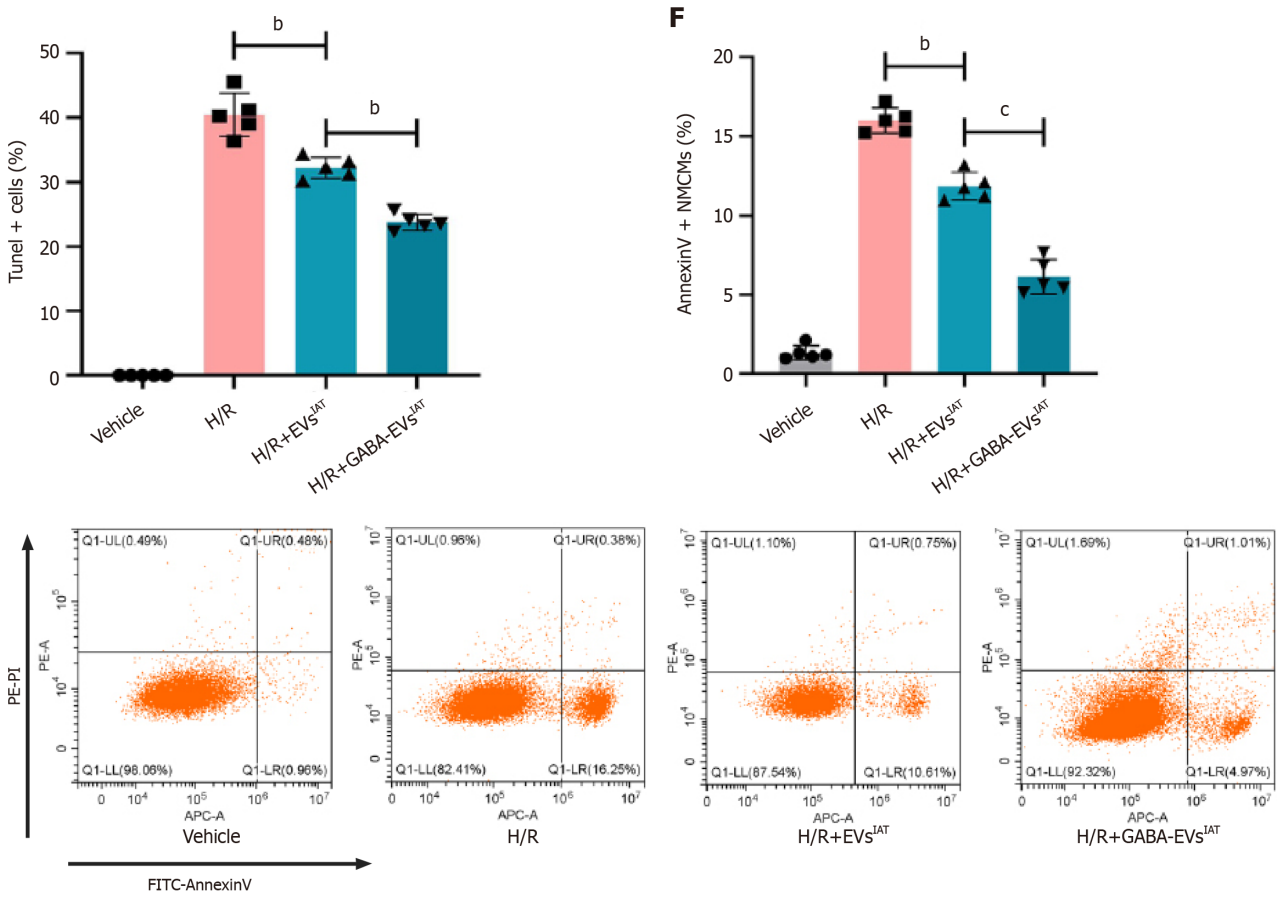


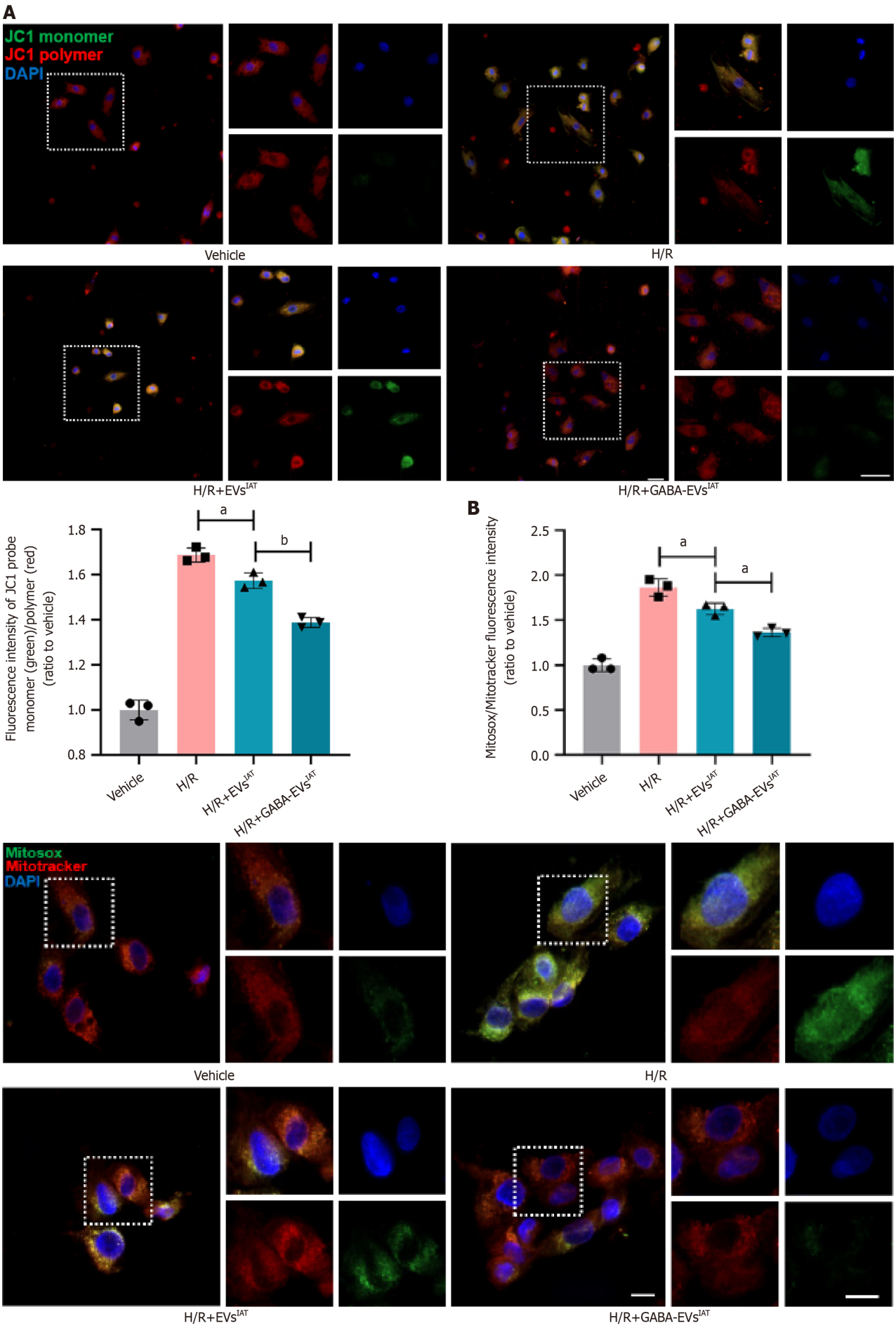
Figure 1 Gamma-aminobutyric acid-induced adipose-derived mesenchymal stem cells from inguinal adipose tissue exhibits potential in mitigating neonatal mouse cardiomyocytes injury under hypoxia and reoxygenation conditions *in vitro*. A: Experiment design. The cardiomyocytes extracted from neonatal mice were categorized into four experimental groups: Vehicle, hypoxia and reoxygenation (H/R), H/R+ extracellular vesicles (EVs) derived from adipose-derived mesenchymal stem cells (ADSCs) from inguinal adipose tissue (IAT), H/R+ gamma-aminobutyric acid (GABA)-induced ADSCs from IAT; B: Experiment design. ADSCs were extracted from the IAT of mice using an enzymatic digestion method. After incubating the ADSCs in complete medium containing 5 μM GABA for 48 h, EVs were extracted; C: Representative micrographs of EVs examined by transmission electron micrographs (scale bars = 500 nm); D: Representative micrographs of PKH67 (scale bars = 200 nm); E: Representative micrographs of isolated neonatal mouse cardiomyocytes stained with terminal deoxynucleotidyl transferase dUTP nick end-labeling (n = 5; scale bars = 200 μm); F: The percentage of AnnexinV+ neonatal mouse cardiomyocytes were calculated using flow cytometry (n = 5). $^{b}P < 0.01$, $^{c}P < 0.001$.

Administration of GABA-EVs^{IAT} mitigates myocardial damage and enhances cardiac function following MIRI

To assess the potential protective effects of GABA-EVs^{IAT} against myocardial damage induced by MIRI *in vivo*, we evaluated cardiac infarct size and AAR in mice that received in situ injections of EVs^{IAT} and GABA-EVs^{IAT} following ischemia/reperfusion (I/R) surgery. As depicted in Figure 3A, AAR/LV values were comparable among the sham, I/R, I/R + EVs^{IAT}, and I/R + GABA-EVs^{IAT} groups (re-ligating the LCA prior to Evans blue staining for AAR/LV calculation). The I/R + EVs^{IAT} and I/R + GABA-EVs^{IAT} groups exhibited significantly reduced infarct size after MIRI compared to the IR group, with the GABA-EVs^{IAT} group demonstrating the most pronounced effect (P = 0.0015 for I/R + EVs^{IAT} *vs* I/R group; P = 0.0002 for I/R + GABA-EVs^{IAT} *vs* I/R + EVs^{IAT} group). Situ apoptosis and serum cardiac markers were assessed using TUNEL assay and enzyme-linked immunosorbent assay in infarct tissues and serum samples respectively (Figure 3B). The degree of in situ apoptosis was significantly improved in both the I/R + EVs^{IAT} and I/R + GABA-EVs^{IAT} groups. I/R + GABA-EVs^{IAT} group exhibited a more remarkable reduction in apoptotic rate compared to the I/R + EVs^{IAT} group. Furthermore, the I/R + GABA-EVs^{IAT} group demonstrated a greater amelioration of serum cardiac markers than both I/R and I/R + GABA-EVs^{IAT} group. The cardiac function was assessed through animal echocardiography, revealing a remarkable enhancement in cardiac contractile function within the IR + GABA-EVs^{IAT} group when compared to both the I/R and I/R + EVs^{IAT} groups (Figure 3C).

GABA-induced miR-21-5p as a potential regulator of TXNIP in GABA-EVs^{IAT}

To explore the mechanism underlying the higher inhibitory efficacy of GABA-EVs^{IAT} on H/R-mediated cardiomyocyte injury and mitochondrial dysfunction, miRNA and mRNA sequencing was performed on EVs^{IAT} , GABA-EVs^{IAT}, and NMCMs exposed to them respectively. A total of 61 miRNAs expressed differently in GABA-EVs^{IAT} compared to EVs^{IAT} and 89 mRNA expressed differently in NMCMs exposed to EVs^{IAT} and GABA-EVs^{IAT}. The top 10 differentially expressed mRNAs and miRNAs, as identified from two sequencing results, were validated for expression using PCR (Figure 4A and B). Among those, miRNA-21-5p, miRNA-23a-3p, miRNA-199a-3p, miRNA-24-3p, miRNA-23-3p, miRNA-34-5p, and


A
JC1 monomer
JC1 polymer
DAPI
Vehicle
H/R
H/R+EVs^IAT
H/R+GABA-EVs^IAT
Fluorescence intensity of JC1 probe monomer (green)/polymer (red) (ratio to vehicle)
a
b
Vehicle
H/R
H/R+EVs^IAT
H/R+GABA-EVs^IAT
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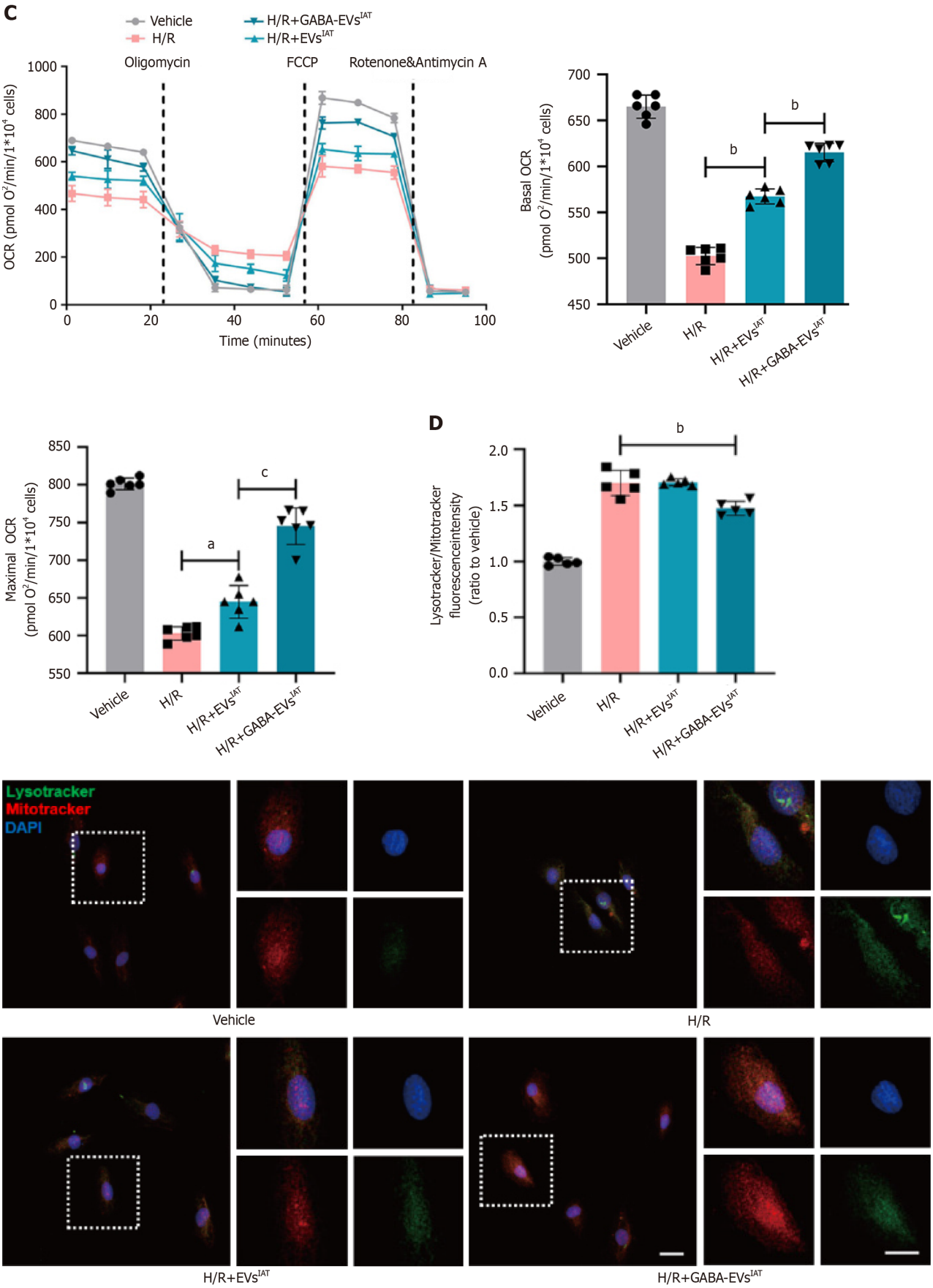
H/R+GABA-EVs^IAT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Time (minutes)
Basal OCR (pmol O2/min/1*10^4 cells)
Maximal OCR (pmol O2/min/1*10^4 cells)
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H/R+EVs^IAT
H/R+GABA-EVs^IAT

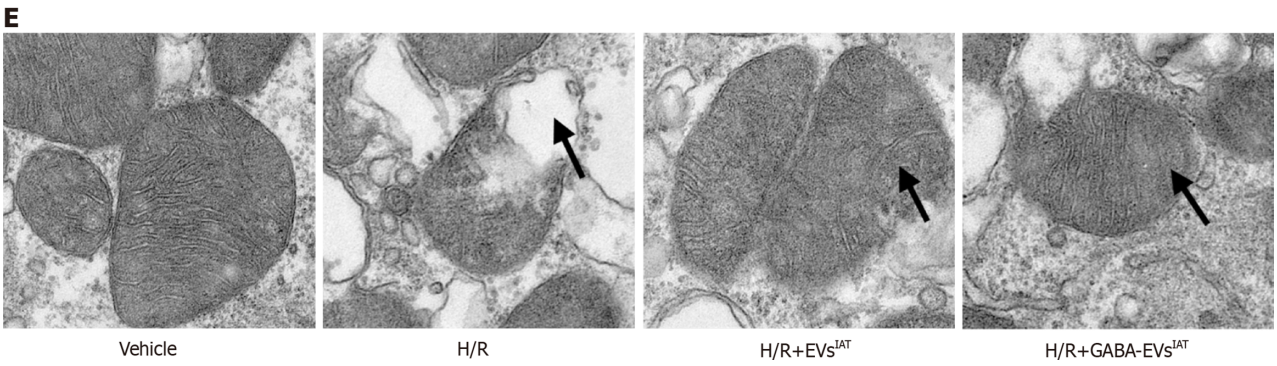


Figure 2 Gamma-aminobutyric acid-induced adipose-derived mesenchymal stem cells from inguinal adipose tissue mitigates neonatal mouse cardiomyocytes mitochondrial oxidative stress levels and enhances mitochondrial function during hypoxia and reoxygenation *in vitro*. A: Representative micrographs of isolated neonatal mouse cardiomyocytes (NMCMs) stained with JC1 probe (n = 3; scale bars = 200 μm); B: Representative micrographs of isolated NMCMs stained with mitosox probe (n = 3; scale bars = 200 μm); C: Real-time oxygen consumption rates (OCRs) and calculated basal and maximal respiration rates in NMCMs (n = 6); D: Representative micrographs of isolated NMCMs stained with mitotracker and lysotracker (n = 5; scale bars = 200 μm); E: Representative micrographs of extracellular vesicles (EVs) examined by transmission electron micrographs (scale bars = 500 nm). $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$. GABA: Gamma-aminobutyric acid; H/R: Hypoxia and reoxygenation; IAT: Inguinal adipose tissue.

miRNA-214-3p were predicted to associate with TXNIP which exhibited the most pronounced decrease by the TargetScan and miRanda algorithms in the Encyclopedia of RNA Interactomes database (Figure 4C). Among the seven miRNAs predicted to bind with TXNIP, miR-21-5p was selected for further study because its upregulation was the most significant. Dual-luciferase reporter assay results showed that the fluorescence ratio was significantly reduced from HEK 293T cells upon co-transfection with miR-21-5p mimics and TXNIP wild-type-3′ UTR compared to the NC and mutant 3′ UTR controls (P = 0.0046) (Figure 4D). These results indicated that miR-21-5p inhibits the translation of TXNIP by targeting the 3′ UTR region of its mRNA.

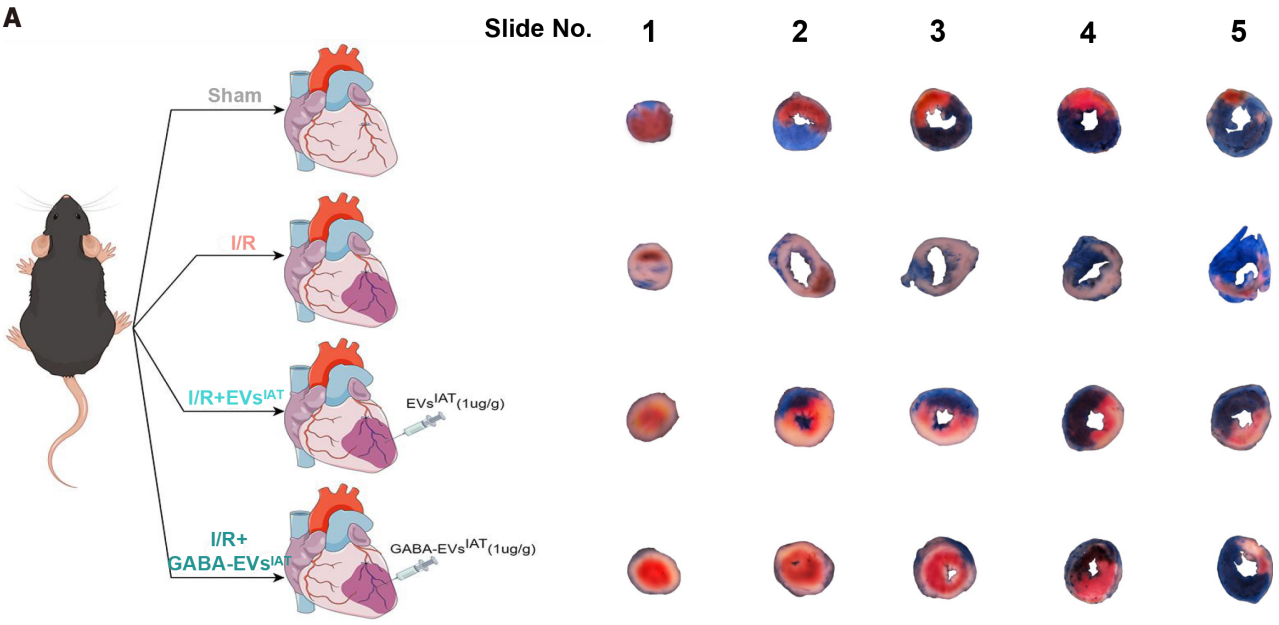
miR-21-5p derived from GABA-EVs^{IAT} regulates TXNIP expression in NMCMs

To clarify TXNIP as the main target of EVs regulation, we assessed the protein expression of TXNIP through western blotting and immunofluorescence in NMCMs. The western blot results (Figure 5A) showed that under H/R conditions, the expression of TXNIP in NMCMs was significantly reduced in the presence of H/R + GABA-EVs^{IAT} compared to the H/R and H/R + EVs^{IAT} groups (P = 0.0008 for H/R + EVs^{IAT} *vs* H/R group; P = 0.0025 for H/R + GABA-EVs^{IAT} *vs* H/R + EVs^{IAT} group). Similarly, immunofluorescence (Figure 5B) also demonstrated the same trend in TXNIP expression (P = 0.0042 for H/R + EVs^{IAT} *vs* H/R group; P = 0.0005 for H/R + GABA-EVs^{IAT} *vs* H/R + EVs^{IAT} group). The EVs of miR-21 KO mice ADSCs were subsequently extracted following GABA induction (GABA-EVs$^{IAT\ miR\text{-}21KO}$) and schematic diagram is presented in Figure 5C. Western blotting and immunofluorescence were employed to investigate whether miR-21-5p serves as the primary regulator of TXNIP in GABA-EVs^{IAT}. After the KO of miR-21, the regulatory influence exerted by GABA-EVs$^{IAT\ miR\text{-}21KO}$ on TXNIP expression significantly diminishes (Figure 5D and E). The aforementioned research findings suggested that miR-21-5p, which is encapsulated within secretory vesicles of ADSCs^{IAT} under the influence of GABA, served as a pivotal molecule governing the expression of TXNIP in NMCMs during H/R pathological conditions.

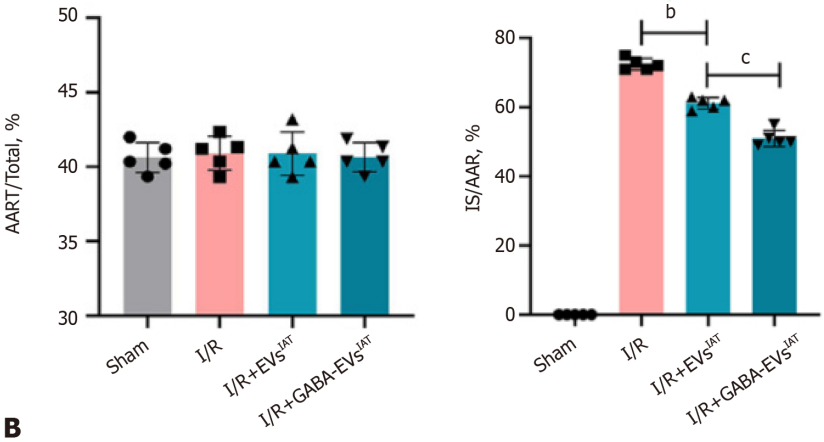
GABA-EVs^{IAT} alleviate MIRI is by regulating NMCMs TXNIP expression through miR-21-5p incorporation

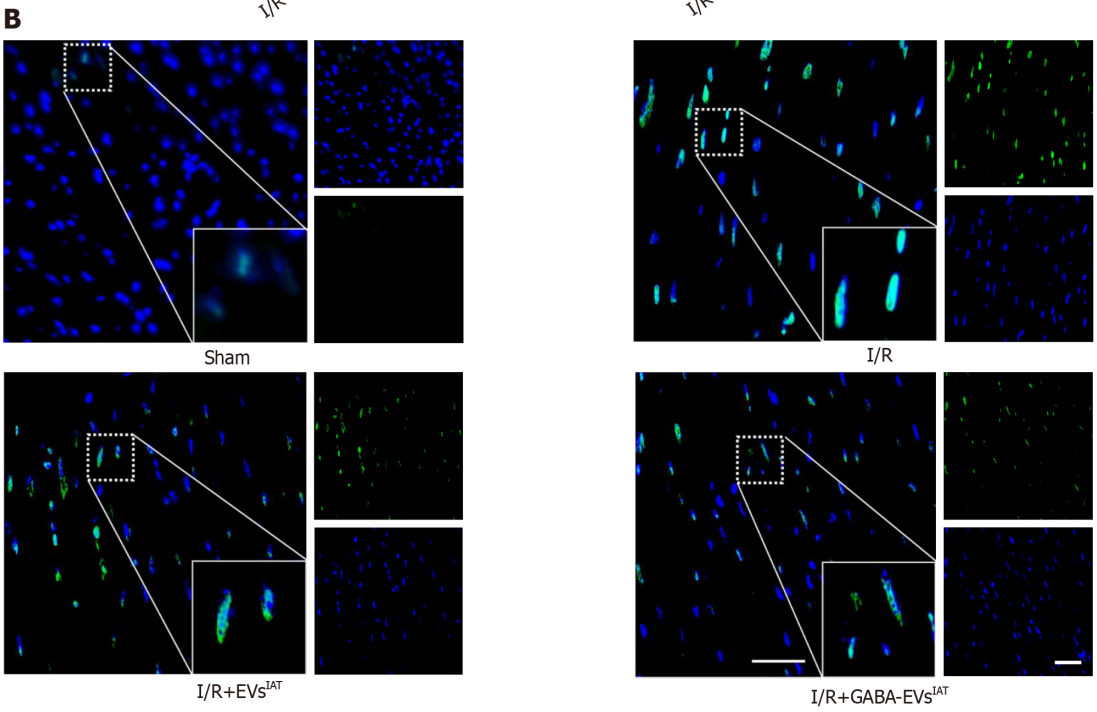
A series of rescue experiments were conducted to elucidate the causal relationship between miR-21 derived from GABA-EVs^{IAT} and its improvement on MIRI by regulating TXNIP. We generated TXNIP KO mice and isolated their NMCMs$^{TXNIP\ KO}$. The schematic diagram in this section can be referred to Figure 6A. The TUNEL detection results indicate that knocking out miR-21 in GABA-EVs^{IAT} significantly weakens the ability of GABA-EVs$^{IAT\ miR21\ KO}$ to protect NMCMs against H/R (P = 0.0006 for H/R + GABA-EVs^{IAT} *vs* H/R + GABA-EVs$^{IAT\ miR\text{-}21\ KO}$ group). However, when TXNIP in NMCMs$^{TXNIP\ KO}$ is knocked out, the above phenotype recovers (Figure 6B) (P = 0.0008 for H/R + GABA-EVs$^{IAT\ miR21\ KO}$ *vs* H/R + GABA-EVs$^{IAT\ miR21\ KO}$ + NMCMs$^{TXNIP\ KO}$ group). Simultaneously, we performed AnnexinV detection, and its findings were in concordance with the observed trend in TUNEL detection (Figure 6C) (P = 0.0005 for H/R + GABA-EVs^{IAT} *vs* H/R + GABA-EVs$^{IAT\ miR\text{-}21\ KO}$ group; P = 0.0003 for H/R + GABA-EVs$^{IAT\ miR21\ KO}$ *vs* H/R + GABA-EVs$^{IAT\ miR21\ KO}$ + NMCMs$^{TXNIP\ KO}$ group).

Subsequently, rescue experiments were conducted to investigate the phenotypic manifestation of mitochondrial function. Evaluation of mitochondrial membrane potential revealed that KO of miR-21 in GABA-EVs^{IAT} significantly attenuated the protective ability of GABA-EVs$^{IAT\ miR21\ KO}$ against H/R in NMCMs. However, this phenotype was restored when TXNIP was knocked out in NMCMs$^{TXNIP\ KO}$ (Figure 6D) (P = 0.0005 for H/R + GABA-EVs^{IAT} *vs* H/R + GABA-EVs$^{IAT\ miR\text{-}21\ KO}$ group; P = 0.0003 for H/R + GABA-EVs$^{IAT\ miR21\ KO}$ *vs* H/R + GABA-EVs$^{IAT\ miR21\ KO}$ + NMCMs$^{TXNIP\ KO}$ group). Furthermore, the oxidative decoupling function (Figure 6E) and levels of oxidative stress (Figure 6F) in NMCMs also demonstrate the aforementioned patterns (P = 0.0007 for H/R + GABA-EVs^{IAT} *vs* H/R + GABA-EVs$^{IAT\ miR\text{-}21\ KO}$ group; P = 0.0023 for H/R + GABA-EVs$^{IAT\ miR21\ KO}$ *vs* H/R + GABA-EVs$^{IAT\ miR21\ KO}$ + NMCMs$^{TXNIP\ KO}$ group). Therefore, the aforementioned findings suggested that GABA-EVs^{IAT} predominantly modulate TXNIP expression in target cells by delivering miR-21-5p, thereby conferring protection against MIRI and promoting mitochondrial homeostasis in NMCMs.



Continuous sections of mouse heart were obtained at a thickness of 1mm/slice





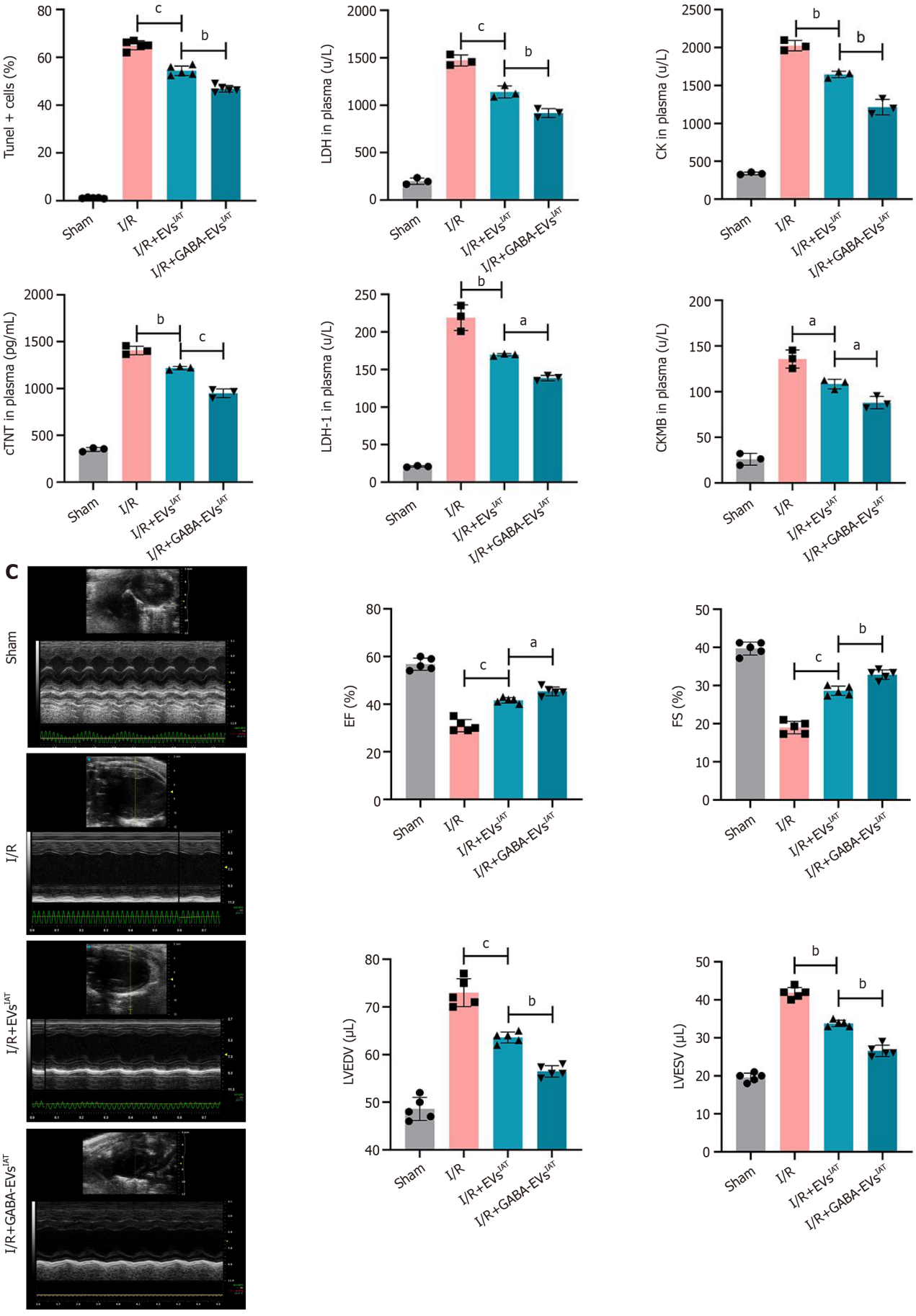


Figure 3 Gamma-aminobutyric acid-induced adipose-derived mesenchymal stem cells from inguinal adipose tissue exhibits potential in

mitigating myocardial injury and improve cardiac function under myocardial ischemia-reperfusion injury conditions *in vivo*. A: Representative graphs of Evans blue/triphenyltetrazolium chloride stain (n = 5); B: Representative micrographs of myocardial tissue section from the infarcted area stained with terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL) (n = 5) and serum cardiac markers degree of mice (n = 3); C: Representative M-mode images of transthoracic echocardiography, and quantification of left ventricular ejection fraction, left ventricular fraction shortening, left ventricular end diastolic volume and left ventricular end systolic volume (n = 5). $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$. AAR: Area at risk; CK: Creatine kinase; cTNT: Cardiac troponin T; CKMB: Creatine kinase-MB; EF: Ejection fraction; EVs: Extracellular vesicles; FS: Fraction shortening; GABA: Gamma-aminobutyric acid; IAT: Inguinal adipose tissue; I/R: Ischemia/reperfusion; IS: Infarct size; LDH: Lactate dehydrogenase; LVEDV: Left ventricular end diastolic volume; LVESA: Left ventricular end systolic volume.

DISCUSSION

EVs are widely recognized as effective nanoscale carriers for targeted delivery of small molecules and nucleic acids to specific tissues or cells, due to their excellent biocompatibility, low immunogenicity, and ability to cross the blood-brain barrier,. Growing evidence also suggests that EVs hold great promise in delivering non-coding RNAs, cytokines, and small-molecule drugs for treating ischemic heart disease[28,29]. However, concerns remain regarding the clinical implementation of EV-based therapies[30,31]. Therefore, our objective was to enhance the therapeutic effectiveness of ADSC^{IAT}-derived EVs against MIRI through a safe GABA-induced method *in vitro* while investigating the underlying mechanisms behind the protective effects exerted by EVs.

White adipose tissue is comprised of subcutaneous fat and visceral fat. Numerous studies have substantiated that subcutaneous adipose, as opposed to visceral adipose, exerts a protective role in neighboring tissues and organs during pathological conditions. ADSCs from the IAT are regarded as one of the key cell types responsible for these effects[17]. It is noteworthy that the benefits conferred by IAT can be transferred to surrounding tissues through adipose transplantation[32]. Paracrine secretion serves as a representative regulatory pathway for these effects, with EVs being one of the most prevalent means of executing paracrine secretion[33]. It is worth noting that previous studies have confirmed the presence of GABAB receptors in ADSCs[17]. Furthermore, ADSCs derived from IAT exhibit a significantly enhanced protective effect on peripheral cells, tissues, and organs under the influence of GABA[34]. These observed disparities in biological functionality can be attributed to alterations in the qualitative and quantitative content of EVs mediated by GABA.

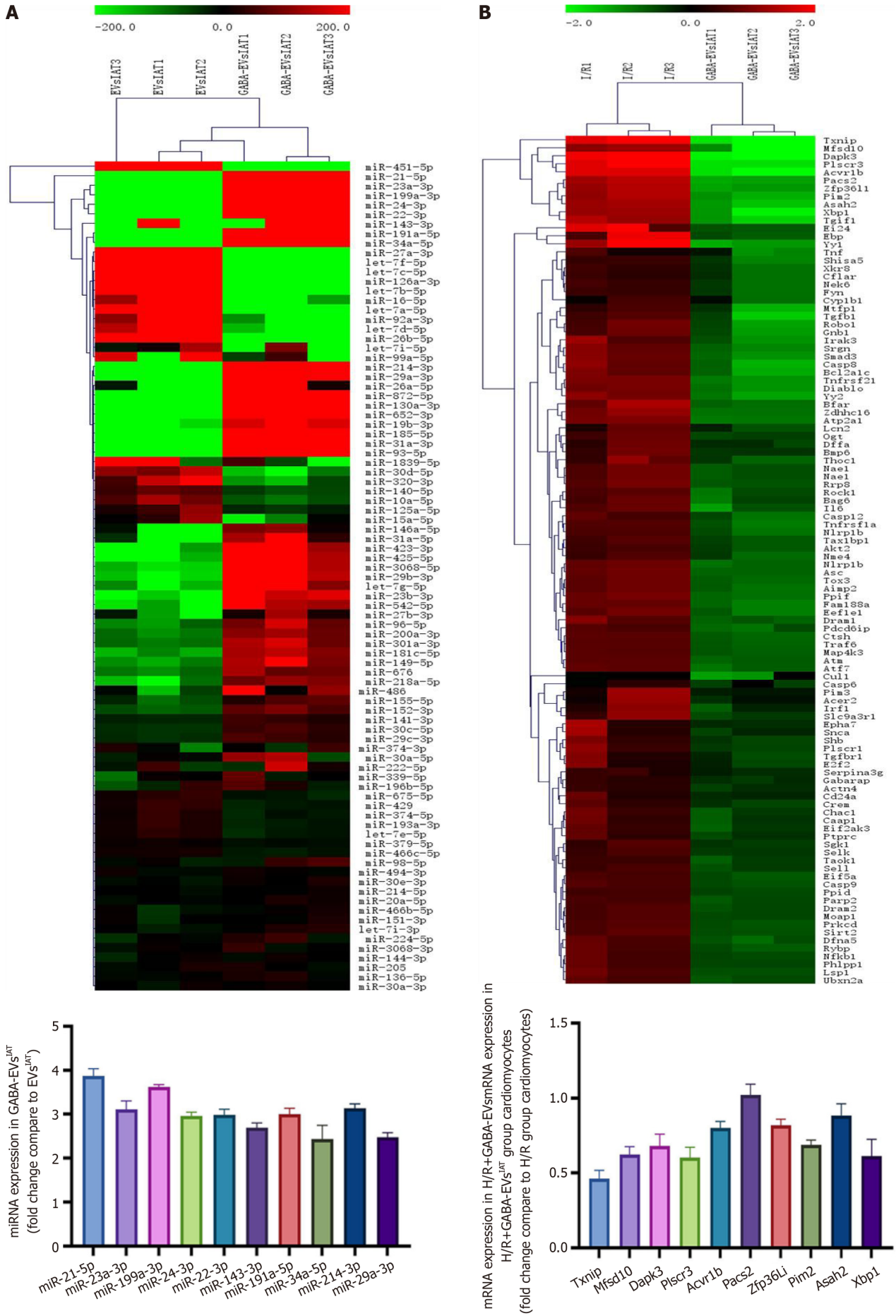
To explore the underlying mechanisms further, we performed multi-omics sequencing on EVs obtained from both ADSCs and cardiomyocytes. Our sequencing analysis revealed a striking discrepancy in abundance for miR-21-5p after administering GABA intervention - its levels showed a remarkable increase. However, the exact role played by miR-21 in GABA-EVs^{IAT} derived from ADSCS remains elusive at present. Consequently, we generated genetically miR-21 KO mice and isolated ADSCs from their IAT. Remarkably, our findings demonstrated that eliminating miR-21 significantly restored the effects induced by GABA treatment. It is noteworthy that the sequencing results of GABA-EVs^{IAT} *vs* EVs^{IAT} demonstrate a significant augmentation in the abundance of multiple miRNAs within the GABA-EVs^{IAT}. However, upon KO of miR-21, the antagonistic impact of GABA-EVs^{IAT} on MIRI and NMCM protection is substantially impeded. This phenotypic alteration suggests that miR-21 serves as a pivotal regulatory miRNA molecule within GABA-EVs^{IAT} for modulating myocardial protective effects under pathological conditions associated with MIRI.

Additionally, transcriptome sequencing was performed on recipient myocardial cells of EVs. As anticipated, the expression of TXNIP exhibited a significant reduction in the GABA-induced EVs intervention group. Dual luciferase reporter gene assays and rescue experiments further validated that TXNIP is among the target genes regulated by miR-21-5p. The induction of ADSCs by GABA leads to a heightened abundance of miR-21 within EVs, thereby suppressing the expression of TXNIP in target cells. This represents one of the fundamental mechanisms through which GABA exerts its protective efficacy in MIRI.

Previous evidence indicates that TXNIP is responsible for coordinating a multitude of cellular responses, including stress, inflammation, and programmed cell death[35,36]. As an inaugural stride in MIRI, the regulation of mitochondrial oxidative stress is deftly governed by TXNIP[37]. As a key reducing molecule that counteracts oxidative stress within mitochondria and as one of the target molecules for TXNIP-induced oxidative stress, TRX2 is known to bind with (apoptosis signal-regulating kinase 1) under stable conditions, thereby inhibiting apoptosis signal-regulating kinase 1-mediated phosphorylation regulation downstream and activating mechanisms such as apoptosis, inflammation, and ferroptosis[38]. This molecular-level change is mainly attributed to the binding and degradation of *TXNIP* mRNA by miR-21-5p derived from EVs, which subsequently alleviates the association between TXNIP and TRX2, rescuing excessive oxidative stress.

In our previous study, we have unveiled EVs derived from ADSCs primarily targeting TXNIP to alleviate myocardial ischemia reperfusion injury[39]. Theoretically and mechanically, TXNIP, interacting with hypoxia-inducible factor-1 alpha, involves the degradation of hypoxia-inducible factor-1 alpha and induces pyroptosis. Thus, by targeting TXNIP, GABA-EVs^{IAT} effectively improve the prognosis of MIRI by means of various intricate mechanisms. It should be duly noted that EVs derived from different sources of ADSCs exhibit distinct biological effects. Presently, it is widely acknowledged that EVs originating from ADSCs^{IAT} possess the remarkable capability to ameliorate pathological conditions such as inflammation, oxidative stress, and insulin resistance[40,41]. Conversely, EVs obtained from ADSCs derived from visceral adipose tissue lack these aforementioned effects[42]. Henceforth, in this study, we procured subcutaneous adipose tissue from the inguinal region in accordance with prior experimental findings.

From a clinical perspective, if the duration of MI surpasses the therapeutic time window of approximately 12 h, during which cardiomyocytes undergo irreversible cell death, even reperfusion therapy proves futile[43,44]. It is postulated that timely initiation of reperfusion therapy is paramount; therefore, interventions aimed at bolstering cardiomyocyte survival


A
-200.0
0.0
200.0
EVsIAT3
EVsIAT1
EVsIAT2
GABA-EVsIAT1
GABA-EVsIAT2
GABA-EVsIAT3
miR-451-5p
miR-21-5p
miR-23a-3p
miR-199a-3p
miR-24-3p
miR-22-3p
miR-143-3p
miR-191a-5p
miR-34a-5p
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miR-16-5p
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miR-92a-3p
let-7d-5p
miR-26b-5p
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miR-224-5p
miR-3068-3p
miR-144-3p
miR-205
miR-136-5p
miR-30a-3p
B
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0.0
2.0
I/R1
I/R2
I/R3
GABA-EVsIAT1
GABA-EVsIAT2
GABA-EVsIAT3
Txnip
Mfsd10
Dapk3
Plscr3
Acvr1b
Pacs2
Zfp36l1
Pim2
Asah2
Xbp1
Tgif1
Bi24
Ebp
Yy1
Tnf
Shisa5
Xkr8
Cflar
Nek6
Fyn
Cyp1b1
Mtfp1
Tgfb1
Robo1
Gnb1
Irak3
Srgn
Smad3
Casp8
Bcl2a1c
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Bfar
Zdhhc16
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Lcn2
Ogt
Dffa
Bmp6
Thoc1
Nae1
Nae1
Rrp8
Rock1
Bag6
Il6
Casp12
Tnfrsf1a
Nlrp1b
Taxlbp1
Akt2
Nme4
Nlrp1b
Asc
Tox3
Aimp2
Ppif
Fam188a
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Dram1
Pdcd6ip
Ctsh
Traf6
Map4k3
Atm
Atf7
Cul1
Casp6
Pim3
Acer2
Irf1
Slc9a3r1
Epha7
Snca
Shb
Plscr1
Tgfbr1
E2f2
Serpina3g
Gabarap
Actn4
Cd24a
Crem
Chac1
Caap1
Eif2ak3
Ptprc
Sgk1
Selk
Taok1
Sell
Eif5a
Casp9
Ppid
Parp2
Dram2
Moap1
Frkcd
Sirt2
Dfna5
Rybp
Nfkb1
Phlpp1
Lsp1
Ubxn2a
miRNA expression in GABA-EVsIAT (fold change compare to EVsIAT)
5
4
3
2
1
0
miR-21-5p
miR-23a-3p
miR-199a-3p
miR-24-3p
miR-22-3p
miR-143-3p
miR-191a-5p
miR-34a-5p
miR-214-3p
miR-29a-3p
mRNA expression in H/R+GABA-EVsmRNA expression in H/R+GABA-EVsIAT group cardiomyocytes (fold change compare to H/R group cardiomyocytes)
1.5
1.0
0.5
0.0
Txnip
Mfsd10
Dapk3
Plscr3
Acvr1b
Pacs2
Zfp36Li
Pim2
Asah2
Xbp1

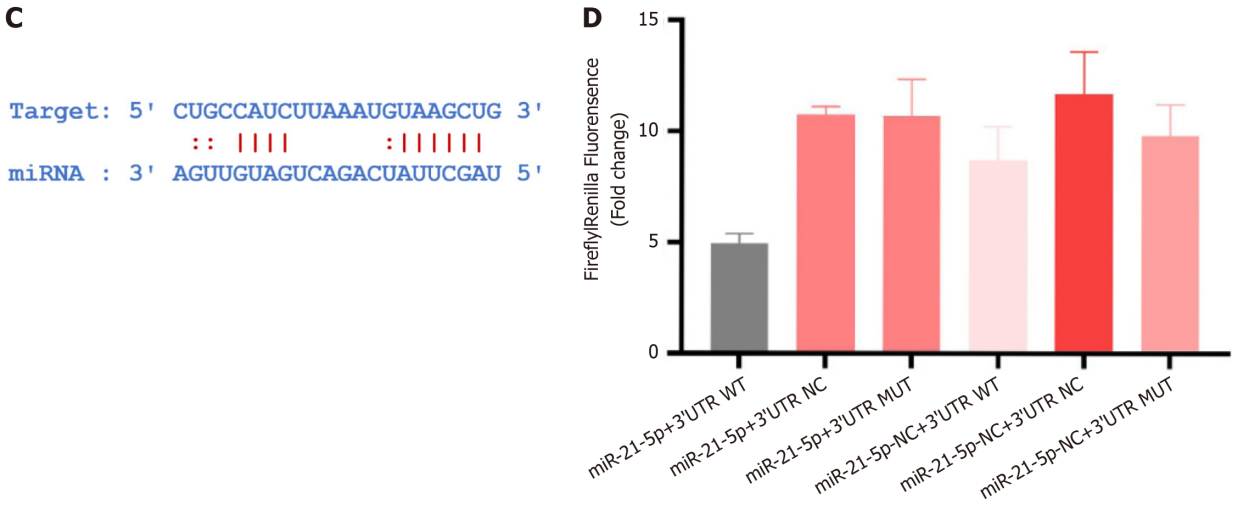
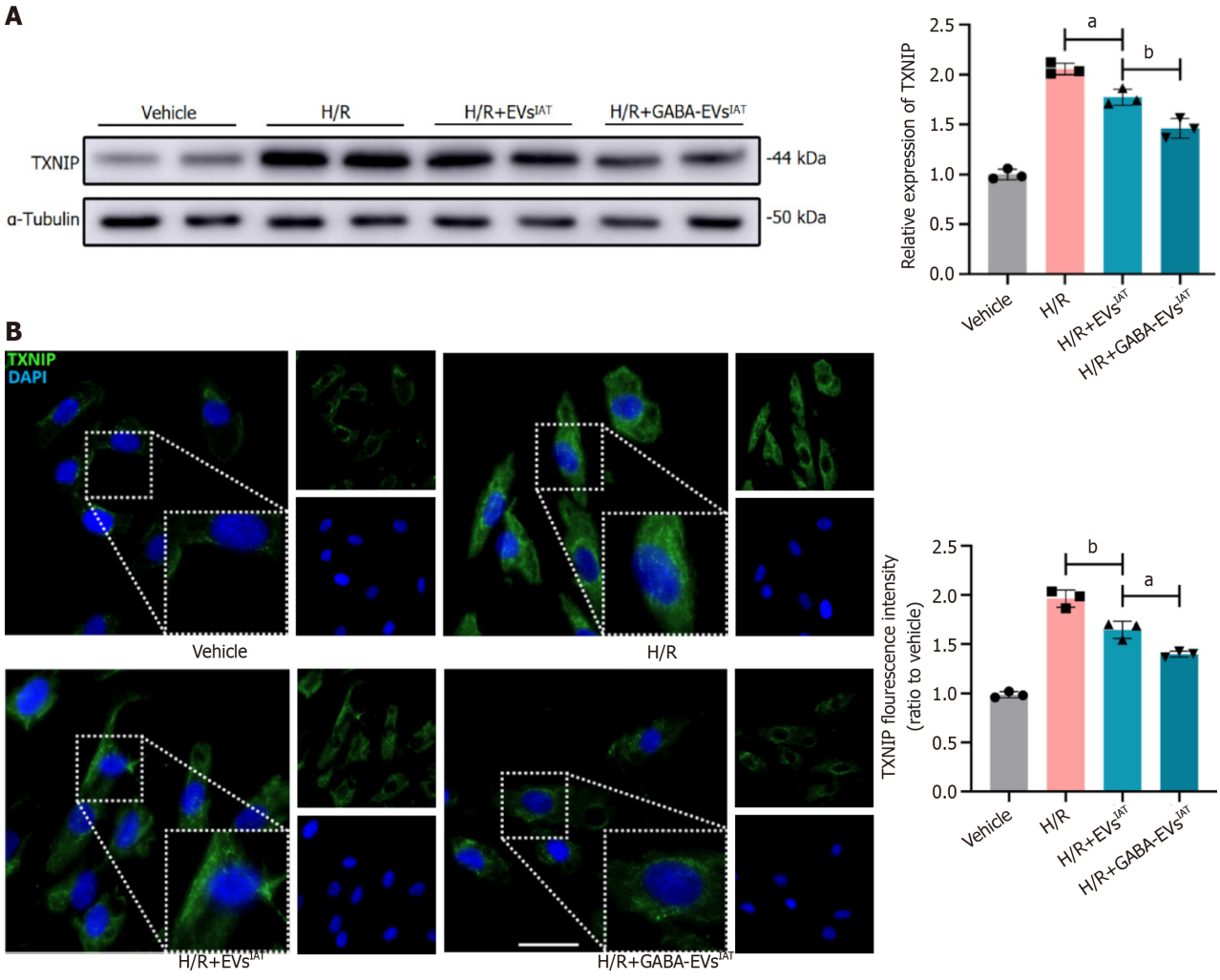


Figure 4 MicroRNA-21-5p derived from gamma-aminobutyric acid-induced adipose-derived mesenchymal stem cells from inguinal adipose tissue regulate thioredoxin-interacting protein expression in mRNA degree. A: MicroRNA (miRNA) sequencing results of gamma-aminobutyric acid (GABA)-induced adipose-derived mesenchymal stem cells (ADSCs) from inguinal adipose tissue (IAT) *vs* extracellular vesicles (EVs)^{IAT}; B: mRNA sequencing results of neonatal mouse cardiomyocytes exposed to GABA-induced ADSCs from IAT; C: Binding sites predicted for the 3' untranslated region (UTR) regions of thioredoxin-interacting protein and miR-21-5p; D: Validation of the binding between thioredoxin-interacting protein and miR-21-5p using a dual luciferase reporter gene assay. H/R: Hypoxia and reoxygenation; miRNA: MicroRNA; MUT: Mutated; NC: Negative control; WT: Wild-type.



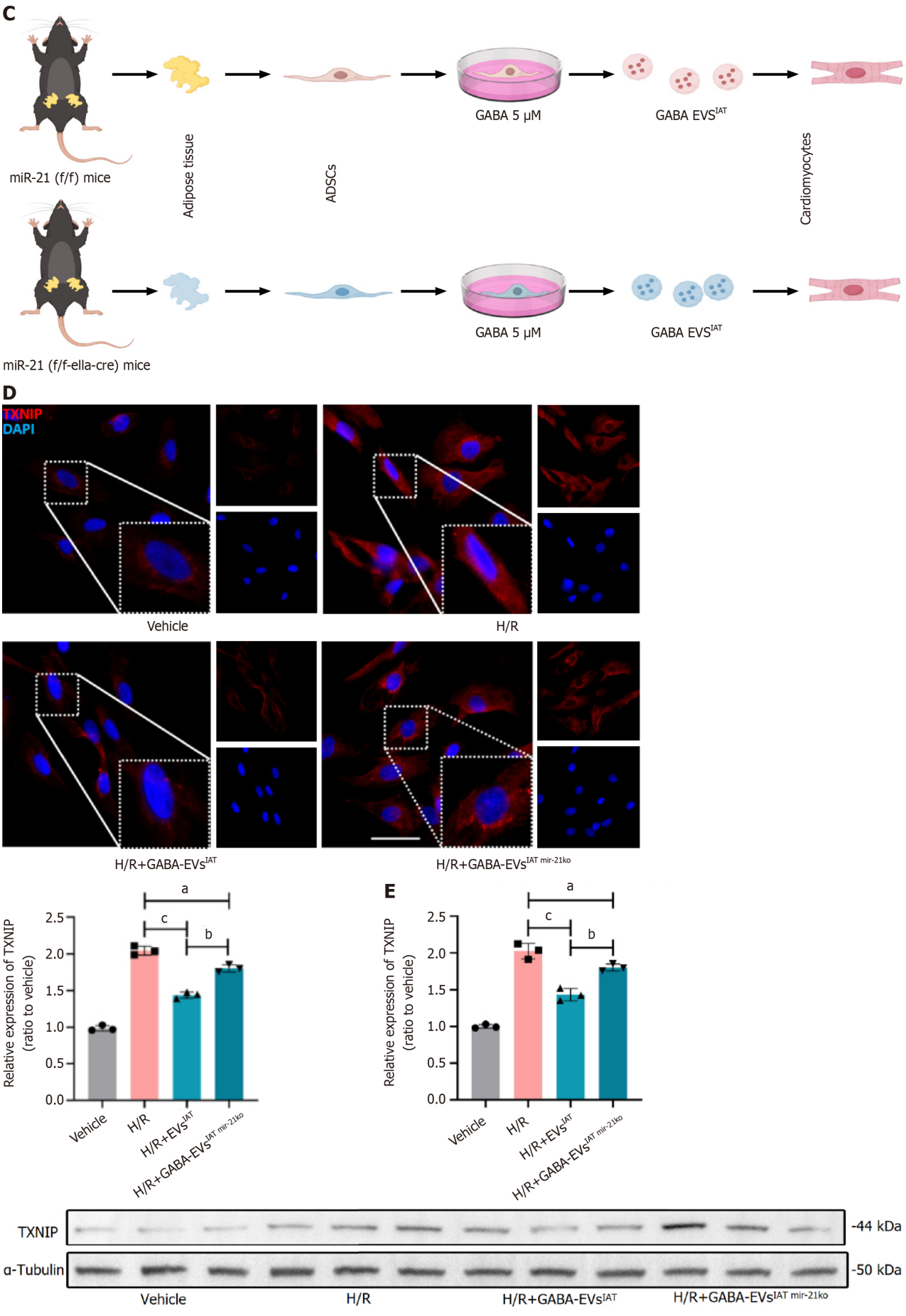


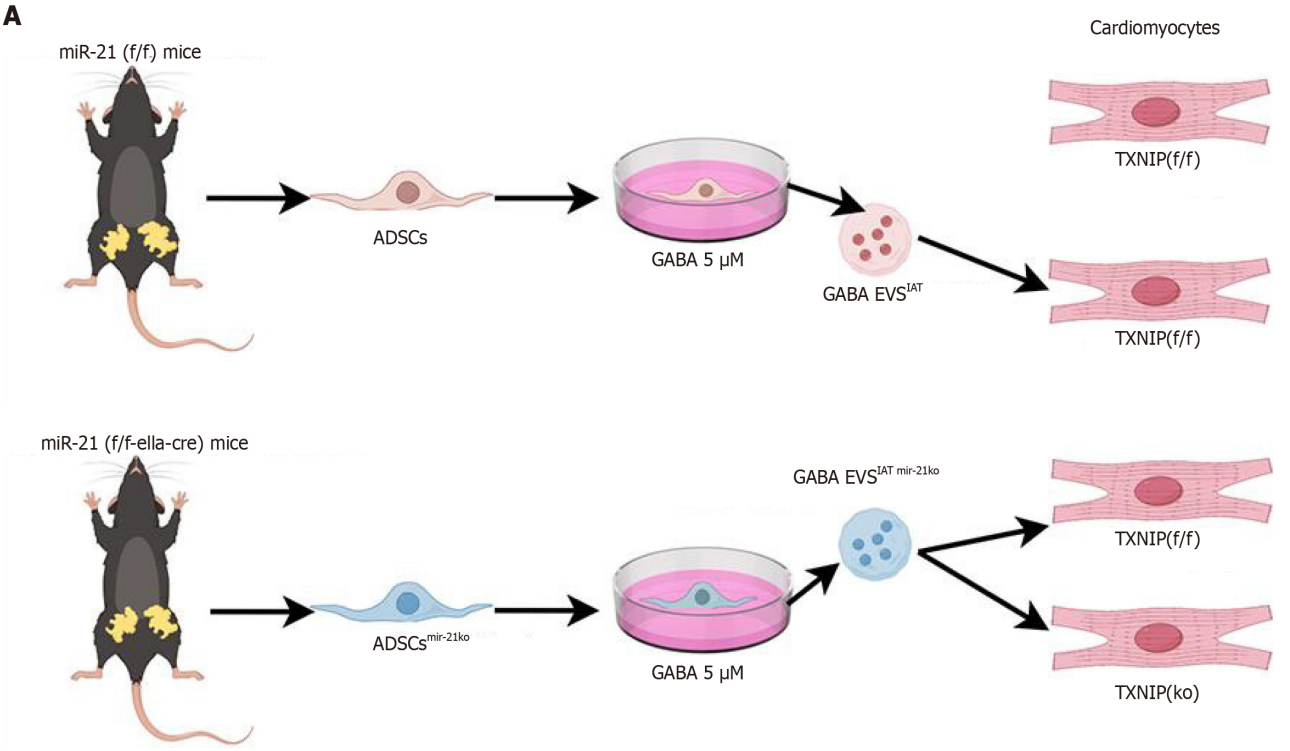
Figure 5 MicroR-21-5p derived from gamma-aminobutyric acid-induced adipose-derived mesenchymal stem cells from inguinal adipose

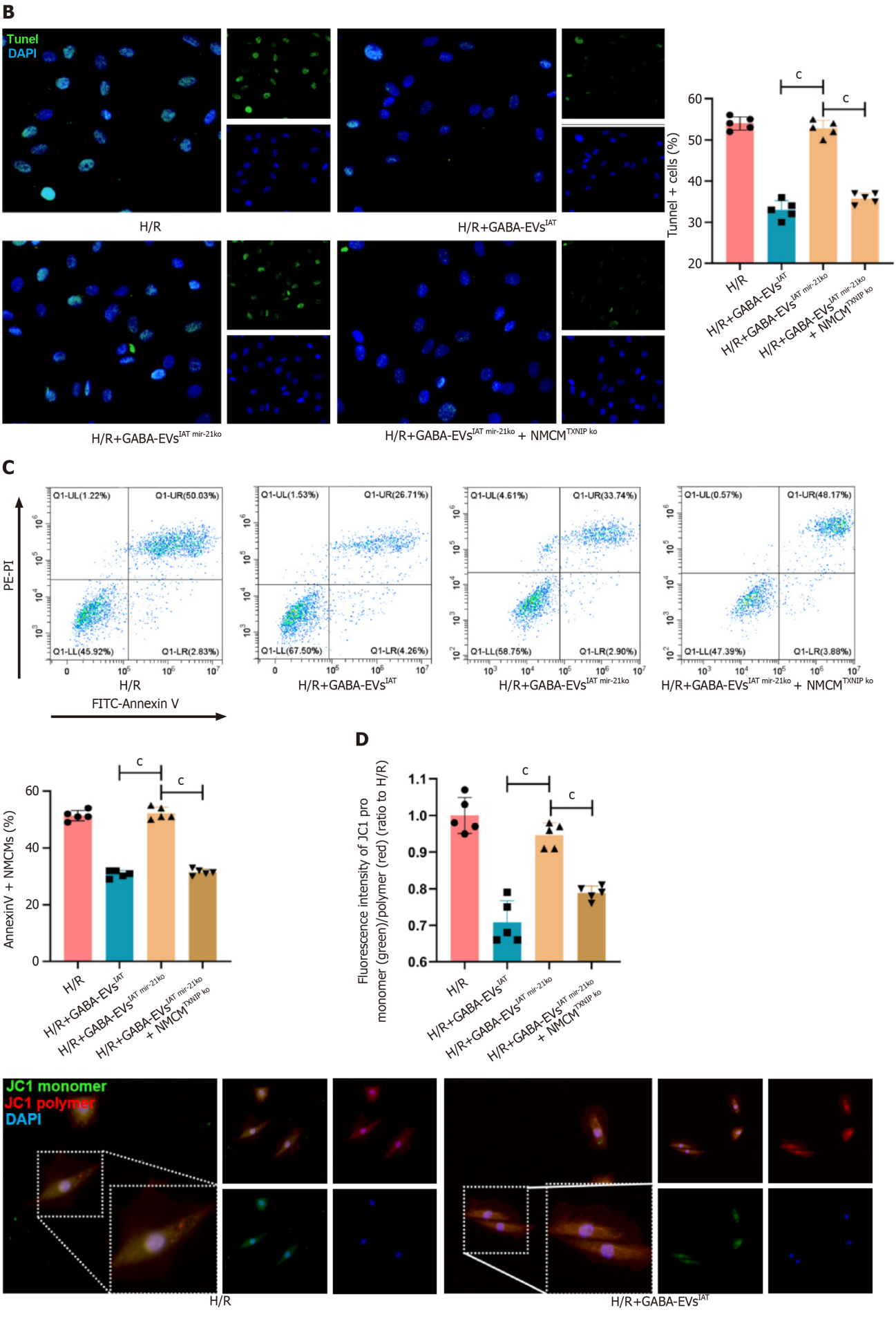
tissue regulate thioredoxin-interacting protein expression in protein degree. A: Representative immunoblots and quantitative analysis of thioredoxin-interacting protein (TXNIP) at the protein level in neonatal mouse cardiomyocytes (NMCMs) (n = 3); B: Representative micrographs of NMCMs stained with TXNIP antibody (n = 3; scale bars = 200 μm); C: Experiment design. Adipose-derived mesenchymal stem cells (ADSCs) were isolated from both miR-21 KO and miR-21$^{f/f}$ mice inguinal adipose tissue (IAT), and extracellular vesicles (EVs) were extracted respectively; D: Representative micrographs of NMCMs stained with TXNIP antibody (n = 3; scale bars = 200 μm); E: Representative immunoblots and quantitative analysis of TXNIP at the protein level in NMCMs (n = 3). $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$. GABA: Gamma-aminobutyric acid; H/R: Hypoxia and reoxygenation.

from the onset of MI until reperfusion therapy become particularly significant. It is assumed that reperfusion therapy can be performed in a fixed time period, then therapies that support cardiomyocytes to survive from onset of MI to reperfusion therapy are particularly significant. Hence, the implementation of early reperfusion strategies for occluded blood vessels and proactive intervention in MIRI are two pivotal treatment approaches aimed at safeguarding a greater number of myocardial cells from succumbing to catastrophic damage following a cardiac event. In conclusion, our study demonstrates that GABA-EVs^{IAT} show a more significant cardioprotection against MIRI than EVs^{IAT}, which partly is attributed to the abundant load of miR-21-5p targeting TXNIP, thereby exacerbating cardiomyocyte mitochondrial oxidative stress levels and facilitating the progression of programmed cell death in cardiomyocytes.

CONCLUSION

In conclusion, our study demonstrated that EVs derived from ADSCs obtained from IAT treated with GABA exhibited significant cardioprotective effects against mitochondrial oxidative stress. These protective effects may be attributed to the ability of EVs to deliver miR-21-5p, which targets TXNIP, thereby regulating the formation of TXNIP-TRX complexes and subsequently enhancing TRX's antioxidant activity (Figure 7 graphical abstract).




B
Tunel
DAPI
H/R
H/R+GABA-EVs^IAT
H/R+GABA-EVs^IAT mir-21ko
H/R+GABA-EVs^IAT mir-21ko + NMCM^TXNIP ko
Tunnel + cells (%)
C
H/R
H/R+GABA-EVs^IAT
H/R+GABA-EVs^IAT mir-21ko
H/R+GABA-EVs^IAT mir-21ko + NMCM^TXNIP ko
C
PE-PI
FITC-Annexin V
Q1-UL(1.22%)
Q1-UR(50.03%)
Q1-LL(45.92%)
Q1-LR(2.83%)
Q1-UL(1.53%)
Q1-UR(26.71%)
Q1-LL(67.50%)
Q1-LR(4.26%)
Q1-UL(4.61%)
Q1-UR(33.74%)
Q1-LL(58.75%)
Q1-LR(2.90%)
Q1-UL(0.57%)
Q1-UR(48.17%)
Q1-LL(47.39%)
Q1-LR(3.88%)
D
AnnexinV + NMCMs (%)
Fluorescence intensity of JC1 pro monomer (green)/polymer (red) (ratio to H/R)
JC1 monomer
JC1 polymer
DAPI
H/R
H/R+GABA-EVs^IAT

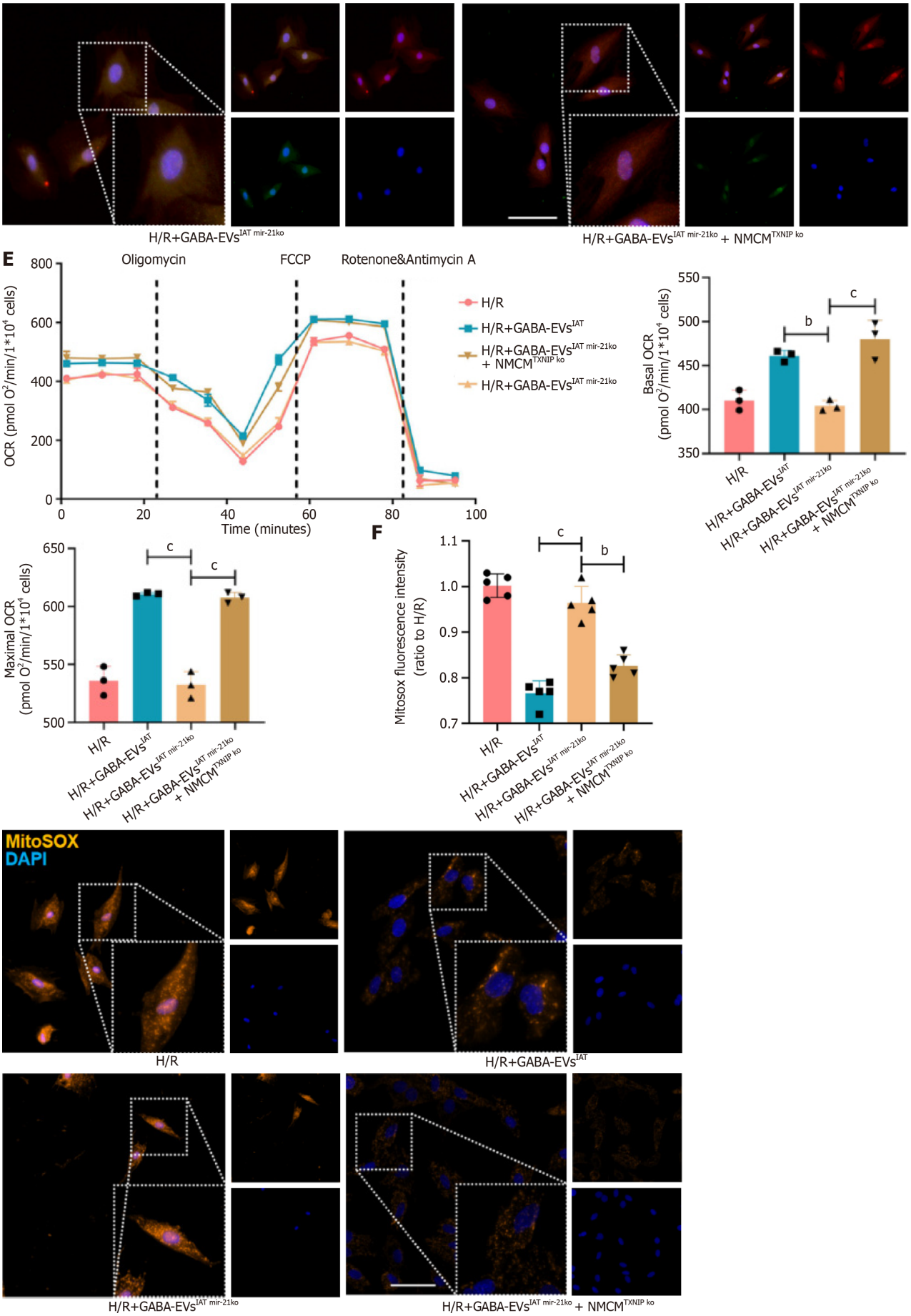


Figure 6 Extracellular vesicles derived from adipose-derived mesenchymal stem cells from inguinal adipose tissue-associated microRNA-

21-5p alleviates hypoxia and reoxygenation-mediated myocardial injury and mitochondrial dysfunctions by downregulating thioredoxin-interacting protein. A: Experiment design. Adipose-derived mesenchymal stem cells (ADSCs) were isolated from both microRNA-21 (miR-21) knockout (KO) and miR-21$^{f/f}$ mouse inguinal adipose tissue (IAT), and extracellular vesicles (EVs) were extracted respectively. Neonatal mouse cardiomyocytes (NMCMs) and NMCMs$^{TXNIP\ KO}$ were respectively treated with gamma-aminobutyric acid (GABA)-induced ADSCs from IAT (GABA-EVs^{IAT}) and GABA-EVs$^{IAT\ miR\ 21KO}$; B: Representative micrographs of NMCMs stained with thioredoxin-interacting protein (TXNIP) antibody (n = 5; scale bars = 200 μm); C: The percentage of AnnexinV+ NMCMs was calculated using flow cytometry (n = 5); D: Representative micrographs of isolated NMCMs stained with JC1 probe (n = 5; scale bars = 200 μm); E: Real-time oxygen consumption rates (OCRs) and calculated basal and maximal respiration rates in NMCMs (n = 3); F: Representative micrographs of isolated NMCMs stained with mitosox probe (n = 5; scale bars = 200 μm). ^{b}P < 0.01, ^{c}P < 0.001, ^{d}P < 0.0001. H/R: Hypoxia and reoxygenation; TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end-labeling.

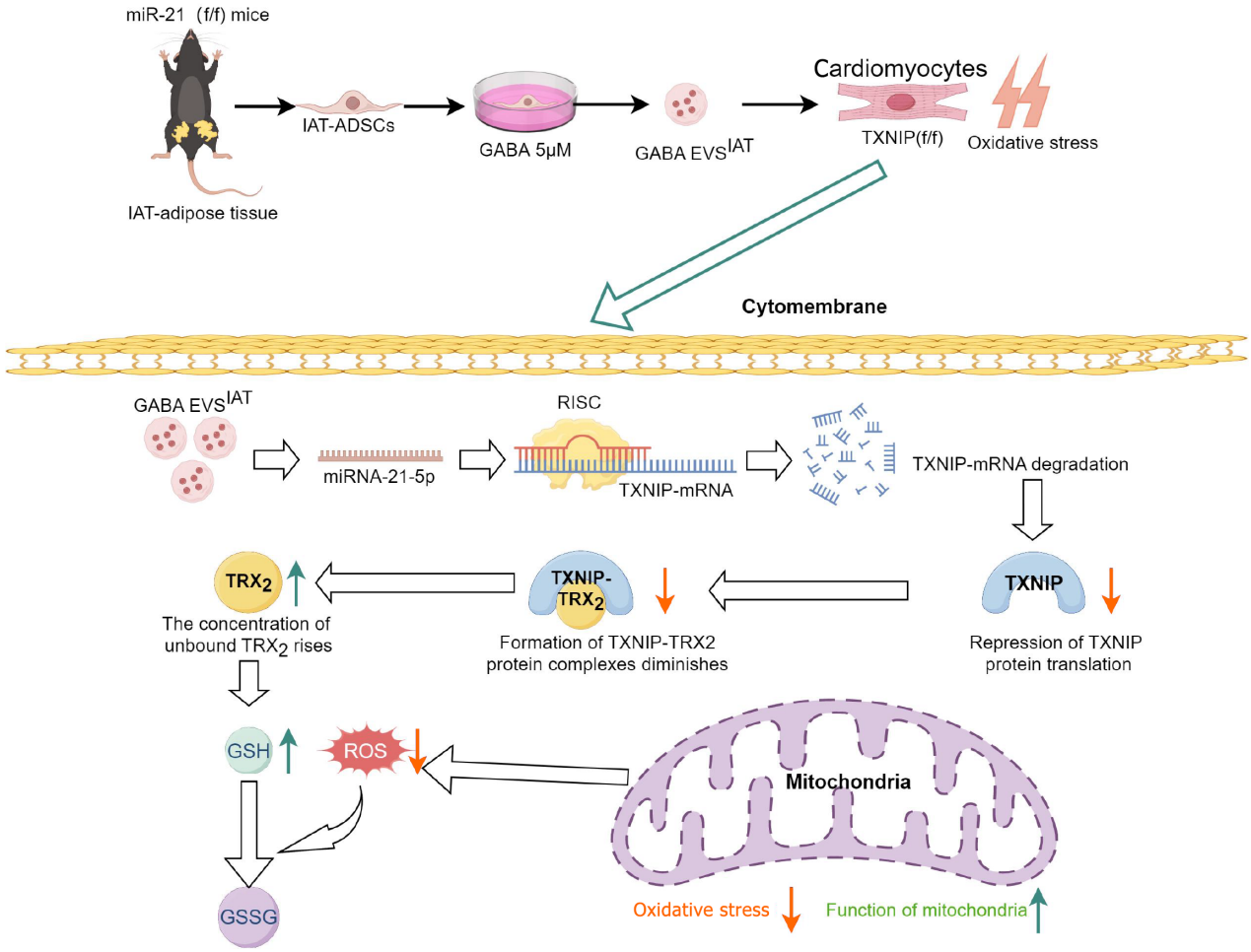


Figure 7 Graphical abstract. Adipose-derived mesenchymal stem cells (ADSCs) derived from subcutaneous adipose tissue, under the influence of gamma-aminobutyric acid (GABA), secrete extracellular vesicles (EVs) abundant in microRNA-21-5p. These EVs possess the ability to impede intracellular thioredoxin-interacting protein (TXNIP) expression in cardiomyocytes, thereby modulating the formation of TXNIP-thioredoxin 2 complex and facilitating thioredoxin 2 involvement in regulating mitochondrial oxidative stress. Consequently, this diminishes the level of mitochondrial oxidative stress in cardiomyocytes and confers protective effects on cardiomyocytes under pathological conditions such as myocardial ischemia-reperfusion injury. GSH: Glutathione; IAT: Inguinal adipose tissue; ROS: Reactive oxygen species; TRX2: Thioredoxin 2.

FOOTNOTES

Author contributions: Wang FD, Ding Y, and Zhou JH contributed equally to this work in performance of the experiments, and review and editing of the manuscript; Zhou E, Zhang TT, Fan YQ, He Q, and Zhang ZQ wrote the paper; Mao CY provided funding for this study; Mao CY, Zhang JF, and Zhou J conceived the study and contributed equally to this work. Wang FD and Ding Y are the co-first authors of this manuscript. Mao CY and Zhou J are the co-corresponding authors of this manuscript. All authors read and approved the final manuscript.

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Institutional animal care and use committee statement: All animal procedures were approved by the Shanghai Ninth People's Hospital Institutional Ethics Committee (Approval No. SH9H-2022-A517-SB) and conducted in accordance with the guidelines of the Directive 2010/63/EU of the European Parliament.

Conflict-of-interest statement: The authors have no conflicts of interest to declare.

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ARRIVE guidelines statement: The authors have read the ARRIVE guidelines, and the manuscript was prepared and revised according to the ARRIVE guidelines.

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LETTER TO THE EDITOR

Emergence of the stromal vascular fraction and secretome in regenerative medicine

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Abstract

Recently, we read a mini-review published by Jeyaraman *et al*. The article explored the optimal methods for isolating mesenchymal stromal cells from adipose tissue-derived stromal vascular fraction (SVF). Key factors include tissue source, processing techniques, cell viability assessment, and the advantages/disadvantages of autologous *vs* allogeneic use. The authors emphasized the need for standardized protocols for SVF isolation, ethical and regulatory standards for cell-based therapy, and safety to advance mesenchymal stromal cell-based therapies in human patients. This manuscript shares our perspective on SVF isolation in canines. We discussed future directions to potentiate effective regenerative medicine therapeutics in human and veterinary medicine.

Key Words: Stromal vascular fraction; Mesenchymal stem cells; Veterinary regenerative medicine; Isolation procedures, Canine model; Secretome

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Core Tip: A recent mini-review highlighted critical steps for optimal stromal vascular fraction (SVF) isolation from adipose tissue, including liposuction refinement, tissue handling, enzymatic digestion, and rigorous quality control for cell viability and purity. This article expanded upon the review by examining the advantages and limitations of SVF isolation, exploring SVF isolation in canine patients, and discussing the future potential of SVF in regenerative medicine.

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TO THE EDITOR

Recently, we read a mini-review article entitled "Understanding and controlling the variables for stromal vascular fraction therapy" by Jeyaraman *et al*[1]. The article provided a comprehensive overview of the critical steps involved in mesenchymal stromal cell (MSC) isolation from stromal vascular fraction (SVF) harvested from adipose tissues. The authors highlighted the significance of each step in determining the quality and quantity of the final product. Furthermore, the authors emphasized standardization, quality control, safety, and ethical consideration before therapeutic application in treating diseases like osteoarthritis.

Including a section on adipose tissue biology, regenerative products, and comparison between autologous and allogeneic SVF sources offers valuable context for understanding the broader implications of MSC isolation. The discussion on anesthetic choices and aspiration steps adds another practical relevance to the article. While the article provided a solid foundation for the SVF isolation and standardization process, other areas could have been further elaborated to enhance the impact of this article.

The authors might have delved deeper into protocol standardization, including specific matrices and benchmarks for accessing MSC quality and quantity. The quantity of stem cells is estimated using stem cell surface markers, which are inaccurate because they identify more abundant committed progenitor cells in addition to stem cells. New technology, such as kinetic stem cell counting, utilizes a label-free technique to quantify specific fractions of stem cells, progenitor cells, and differentiated cells in a biological sample[2]. Precise quantification of stem cells is important in determining the dosage of therapeutic tissue stem cells like hematopoietic stem cells[3]. In a similar fashion, a specific fraction of tissue-specific MSCs can be quantified in SVF harvested from adipose tissue collected from various sites.

This mini-review primarily focused on technical aspects of MSCs isolation. Expanding the utility of MSCs derived from the SVF for clinical applications in other diseases would have strengthened the impact of the article. In addition, given the increasing interest in regenerative medicine, a brief discussion on the regulatory landscape of MSC-based therapy in India would have also been quite relevant.

PERSPECTIVE ON THE CANINE SVF AND SECRETOME

The focus of the article on human adipose tissue is commendable. Expanding the scope to include the isolation of SVF from model animal tissues like canine adipose tissue would have significantly enhanced the relevance to a broader audience. Canine models are frequently used in biomedical research and advancements in canine regenerative medicine. We have observed in the canine model that the adipose tissue harvested from different sites has different proportions of stem cells, of which the peri-ovarian region was the best site for adipose tissue harvest, consistent with the observation from the literature[4]. While human and canine MSCs share many core characteristics, there are also species-specific differences. For instance, canine MSCs exhibit a moderate expression of surface markers like CD90, CD73, and CD166 compared to their human counterparts[5]. Our unpublished data suggest that canine SVF has a faster osteogenic differentiation potential, reaching maturity in 16 days compared to 21 days for human SVF. While the immunomodulatory properties of human SVF, including modulation of T cells and B cells, are well-established, research on the immunomodulatory effects of canine SVF remains limited.

The future of SVF therapy holds immense promise. While currently utilized as a heterogeneous cell population, the potential to optimize its therapeutic efficacy lies in further understanding and manipulating its constituent cells by expansion. One exciting avenue of research involves culturing SVF in the presence of specific compounds to increase the stem cell fraction. Nucleoside analogs, such as xanthosine, promote the proliferation of stem cells from diverse tissues, including canine SVF (unpublished data), rat liver stem cells[6], and bovine and goat mammary stem cells[7,8]. However, the precise molecular mechanisms underlying their actions remain to be fully elucidated.

By identifying and utilizing growth factors, cytokines, or other bioactive molecules, it may be possible to expand the stem cell component within SVF selectively. This could lead to the development of more potent and targeted cell therapies. SVF may present several potential risks and challenges; firstly, the isolation and processing of SVF can introduce microbial contamination or damage to the cells, affecting their viability and function. Secondly, there is a risk of immune reactions, especially allogeneic administration, though MSCs are less immunogenic and safe for therapeutic applications in humans and canines. Additionally, the long-term safety and efficacy of canine SVF therapy remain unknown. Furthermore, the standardization and quality control of canine SVF preparations can be challenging, as the composition and characteristics of SVF can vary widely depending on the source and isolation method.

Another key aspect that needs to be determined is the regulatory and ethical issues. The differences in regulatory and ethical issues between canine SVF and human SVF should be carefully considered when formulating future treatment strategies. The use of human SVF is subject to stricter regulations and ethical guidelines. The National Guidelines for Stem Cell Research in India, as of today, prohibit stem cell therapy, except hematopoietic stem cell therapy for blood

disorders. All other stem cell therapies, including SVF, remain limited to investigational and clinical trial levels after obtaining necessary permissions. Canine SVF, on the other hand, may have less stringent regulations, mainly when used in veterinary medicine. Rules for the use of canine stem cells are yet to come. Ethical considerations, such as the welfare of animals and the potential for unintended consequences, should be carefully addressed while working with canine SVF.

The extraction and quality assessment of canine and human SVF requires careful consideration due to inherent species-specific differences. While both canine and human SVF are derived from adipose tissue, the anatomical location and quality of the tissue can vary between species, potentially affecting the yield and composition of the SVF. The optimal isolation methods for canine and human SVF may differ due to variations in cell surface markers, adhesion properties, and other cellular characteristics. Finally, the norms of the International Society for Cellular Therapy outline general guidelines for the ethical and scientific conduct of stem cell research and clinical applications and apply to both species. In addition to general standards, species-specific guidelines may apply to the production and use of canine SVF. Adherence to good manufacturing practices and principles remained essential for ensuring the quality and safety of SVF products for clinical use.

Another promising strategy involves harnessing the power of the stem cell secretome. This complex mixture of soluble factors, including growth factors, cytokines, and extracellular vesicles, is secreted by stem cells and may play a vital role in tissue repair and regeneration[9], cancer[10], and many other disease conditions. Generating a secretome for novel therapeutic agents would mitigate the challenges associated with allogeneic cell transplantation. Moreover, the secretome provides: (1) Direct administration at the site of injury to promote tissue repair and reduce inflammation; (2) The ability to encapsulate into biocompatible carriers to improve its stability and target delivery; and (3) The development of the synthetic or recombinant version of these molecules for therapeutic use by characterizing components of the secretome.

CONCLUSION

The standardization of SVF isolation is essential for improving the effectiveness of stem cell therapy. Cell-based treatment outcomes may differ due to the differences in tissue-harvesting site isolation protocols, including enzymatic digestion time, centrifugation speed, and many other variables. Harnessing the potential of the stem cell secretome and investigating canine models offer promising avenues for future advancements in SVF-based therapies.

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