Exosomes for Cardioprotection: Are We Ready for Clinical Translation?

Envoy of the European Commission. It also drew on significant input from the Scientific Council of the ERC.

An implementation task force, led by John-Arne Røttingen (RCN) and David Sweeney (UKRI), will now collaborate with other stakeholders and work towards the swift and practical implementation of these principles.

Source: https://www.scienceeurope.org/coalition-s/.

The 10 Principles of Plan S

The key principle is as follows:

'After 1 January 2020 scientific publications on the results from research funded by public grants provided by national and European research councils and funding bodies, must be published in compliant Open Access Journals or on compliant Open Access Platforms'.

In addition:

- Authors retain copyright of their publication with no restrictions. All publications must be published under an open license, preferably the Creative Commons Attribution Licence CC BY. In all cases, the license applied should fulfil the requirements defined by the Berlin Declaration;
- The Funders will ensure jointly the establishment of robust criteria and requirements for the services that compliant high-quality Open Access journals and Open Access platforms must provide;
- In case such high quality Open Access journals or platforms do not yet exist, the Funders will, in a coordinated way, provide incentives to establish and support them when appropriate; support will also be provided for Open Access infrastructures where necessary;
- Where applicable, Open Access publication fees are covered by the Funders or universities, not by individual researchers; it is acknowledged that all scientists should be able to publish their work Open Access even if their institutions have limited means;
- When Open Access publication fees are applied, their funding is standardized and capped (across Europe);
- The Funders will ask universities, research organizations, and libraries to align their policies and strategies, notably to ensure transparency;
- The above principles shall apply to all types of scholarly publications, but it is understood that the timeline to achieve Open Access for monographs and books may be longer than 1 January 2020;
- The importance of open archives and repositories for hosting research outputs is acknowledged because of their long-term archiving function and their potential for editorial innovation;
- The 'hybrid' model of publishing is not compliant with the above principles

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Introduction

In the early 2000s, the concept of cell transplantation for myocardial regeneration moved from bench to bedside within just 1 year.[1,2] Almost 20 years later, we are facing a large number of clinical trials that utilized various stem and progenitor cell preparations with widely contradictory results.[3] Furthermore, a meta-analysis revealed that the study outcomes were heavily biased towards more positive outcomes by inferior methodological quality of study design and absent quality controls for the cells used.[4] Based on the posted results, it remains questionable that currently available cell-based therapies result in true myocardial regeneration.[5]

There is some consensus that in the setting of acute myocardial infarction, remodelling of the infarct border zones may be prevented, resulting in a less compromising myocardial scar.[5] Adult and neonatal progenitor cells alike are believed to exhibit their cardioprotective potential via a pro-angiogenic, anti-fibrogenic, and immune modulatory secretome.[6–8] However, this effect is dependent on retention, source, and quality of stem cells used.[8]

The secretome of cardioprotective cell preparations contain various cytokines as well as extracellular vesicles (EVs).[9] Extracellular vesicles are commonly categorized into three groups based on their genesis: microvesicles, apoptotic bodies, and exosomes.[10] The latter hold a unique miRNA and protein profile that convey a variety of cytoprotective, immune-modulatory, and pro-angiogenic signals *in vitro* and *in vivo*.[6,11] Especially results from pre-clinical experiments in rodent models of myocardial infarction indicate that exosomes promote cardioprotection to a similar degree as their parent cells.[12,13] These findings sparked a series of larger animal trials, two of which have recently posted results[14,15] indicating that clinical testing of exosome may be imminent.

In lieu of the experiences with progenitor cell preparations it is important to fully validate exosome preparations prior to use in clinical trials. Important questions regarding the cardioprotective potential of

exosomes in humans as well as dose, duration and delivery of exosomes are still not fully understood and characterized.

Exosomes in cancer therapy—what can we take from that experience?

Outside of cardiovascular applications, exosomes have already been utilized in clinical trials (*Table 1*). In cancer research, exosomes are used as diagnostic makers as well as therapeutic agents. The idea of using exosomes as tumour vaccines dates back to 1998.[16] Here, dendritic cell-derived exosomes were used as antigen carrying vehicles, which direct a specific auto-immune response to cancer cells via cytotoxic T-cells. In other experiments, clinical grade exosomes were designed to deliver Kras inhibiting siRNAs to pancreatic tumour cells.[17,18] The methodological aspects of some of these studies may serve as blueprints for engineering exosomes for cardioprotection. Our knowledge about exosomes is however still very limited and many aspects of the biology of exosomes is still debated.[19,20]

It is therefore imperative that the mechanism of action (MoA), the MoA defining phenotype, and biological activity of engineered exosomes is known and modes for detecting these three attributes are available. Similar to the adult stem cell hype in the early 2000's many potential therapeutic benefits are attributed to exosome preparations without the necessary level of evidence.[4] In cardiovascular research, recent consensus statements by Coumans *et al.* and Sluijter *et al.* are an impressive basis for defining such criteria and may prevent similar errors from the past.[19,21]

Most consensus statements on the clinical use of extracellular vehicles published in recent years mention the following limitations and challenges:

(1) Current isolation methods are labour intensive and show low efficiency.
(2) The mechanism of action (MoA) of native exosomes is often not well understood.
(3) Characterization is often limited to phenotype and lacks functional assessment.
(4) Repetitive treatments or deposits (e.g. matrices) may be necessary to ensure full therapeutic effect.

As mentioned above, some published studies utilized large-scale exosome production for human trial or large animal studies (*Table 1*). Especially studies in the field of oncology may serve as great examples for study planning and execution. One study that stands out was conducted by Nathalie Chaput *et al.*[17,22] This multicentre trial investigated the potential of dendritic cell-derived exosomes as tumour vaccines in the setting of non-small-cell lung cancer. Based on an optimization study for production of GMP-grade (good manufacturing practices) exosomes they have set up specific quality controls to ensure reproducibility of their experiments.[22] This included phenotype characterization as well as functional assessment *in vitro*. The characterization of phenotype was specified by detection of MHC Class I and II molecules that are not always found in the minimal criteria for exosome characterization but play an important role during antigen presentation in patients during vaccination.[22] In addition, exosomes induce dendritic cell mediated activation of tumour associated antigen specific cytotoxic T-cells in co-culture to ensure functionality of exosomes.

Table 1 Selection of clinical and pre-clinical studies that have utilized exosomes in a larger scale

Study					Exosome quality control		
PMID/ NCT	Therapeutic function	Disease/ model	Number of subjects	Outcomes	Exosome source	Exosome production/ modification	Exosome characterization	Exosome functionality
18362931	Cancer vaccine	Colorectal cancer	40	No major adverse events	Autologous ascitis	UF, UC, SG	EM, WB, PC	None
12379326, 27141373, NCT01159288	Cancer vaccine	Non-small-cell lung cancer	22	Primary endpoint not met	Allogeneic DCs	Tumorantigen exposure of DCs prior to isolation, UC, DF, SG+UC, DF	FACS, ELISA, PC, FA	T cell stimulation was tested with TAA-loaded Dex
24445866	Treatment of graft vs. host disease	Graft vs. host disease	1	Alleviation of symptoms	Allogeneic BM-MSCs	PEG-precipitation, UC	WB, NT, EM, FA	Patient PBMC and NK activation *in vitro*
28158410	Cardioprotection during AMI	Porcine model of AMI	22	Decrease in scar size	Human CDCs	UC, UF, PEG	NT	None
28377922	Immune modulation	Porcine model of synovitis		Reduction of inflammation	Porcine BM-MSCs	UF	PC, NT, FACS	None
29600288	Cardioprotection during AMI	Porcine model of AMI	12	Reduction in remodelling	Human CDCs	PEG, C	none	None

BM-MSCs, bone marrow-derived mesenchymal stem cells; CDCs, cardiosphere derived cells; DCs, dendritic cells; DF, diafiltration; EM, electron microscopy; Exo, exosomes; FA, functional analysis; NK, natural killer cells; NT, nano tracking analysis; PBMC, peripheral blood mononuclear cells; PC, measurement of protein concentration by BSA-assay; SG, sucrose gradient centrifugation; TAA, tumour associated antigen; UC, ultracentrifugation; UF, ultrafiltration; WB, western blot.

Furthermore, the researchers developed multiple assays that allowed them to test their hypothesized MoA, as seen by the longitudinal *in vitro* studies performed on participant's specific immune cells. In the aforementioned study over 10% of exosome isolation had to be discarded because they didn't meet the quality criteria. This highlights the importance of thorough testing to ensure high evidence and reproducibility of study outcomes.

Why we should not rush into clinical trials quite yet

To this day, the cardioprotective potential of CDC (cardiosphere derived cells) derived exosomes has been tested in two large animal studies by the group of Marbán *et al.*[14,15] The studies demonstrate that direct intramyocardial injection is superior to intracoronary injection and that exosomes may have the potential for cardioprotection after myocardial infarction. Additionally, the preservation of fibre helix architecture measured by magnetic resonance imaging is a promising new indirect outcome parameter for efficacy of treatment. These studies set a foundation for future pre-clinical studies and ultimately clinical trials. Necessary steps towards that path include a more in-depth characterization of exosomes used in pre-clinical trials. The characterization should aim to identify the cardioprotective properties of exosomes.[10] To date, there is no generally accepted method for testing the cardioprotective potential of exosomes.

Future research should focus on defining and developing such methods to ensure reproducibility and quality of exosomes. As an example, the inhibition of cardiac fibroblast proliferation could be functionally tested *in vitro*.[7] Mesenchymal stem cell (MSC) conditioned medium is known to suppress fibroblast proliferation and reduce expression of matrix metalloproteinases.[7,23] Both play an essential role in overshooting fibrosis in the border zone of the infarct. The inflammatory phase after infarction also has a great impact on the ultimate scar size.[24] During this phase, neutrophils, T-cells and macrophages home-in to the injured tissue and create a pro-inflammatory environment. Resolution of this phase is imperative to proceed towards the remodelling phase, which is partly mediated by the switch of macrophages from an M1 state to an M2 state[24] and is influenced by MSCs in animal models and *in vitro* studies.

A study by Monguió-Tortajada *et al.*, which utilized well defined and purified exosomes from umbilical cord demonstrated that contamination of exosome preparations with other EVs and proteins directly impacts the immune modulatory potential of exosomes.[25] All assays were performed *in vitro* and can be performed in patient-specific haematopoietic cells. Monguió-Tortajada *et al.* showed that only the pure exosome fraction has a dose dependent impact on T-cell suppression, whereas macrophage polarization is not affected. This observation highlights a common issue seen with current exosome protocols. Depending on isolation protocols applied, the composition of exosome preparations can be impure and not well defined.[20,26] The majority of methods currently available are solely based on discrimination of particle size and are therefore rather non-specific.[19] Immunoprecipitation assays are available and could potentially help in the future to isolate more specific exosome populations.[27,28]

Most clinical and animal studies rely on methods for exosome purification that yield varying purities of exosome solutions (*Table 1*). The most common contaminations in cell culture supernatants that utilize foetal bovine serum (FBS) are lipoproteins, and foreign miRNAs.[29] For clinical applications, it is imperative to develop optimal xeno and serum-free media formulations that are compatible with exosome production. Removing serum from media may alter the quality and functionality of exosomes.[19] The mechanisms that alter exosome secretion by parent cells in response to microenvironmental changes are still not well understood. This indicates that protocols developed under non-GMP conditions for pre-clinical studies have to be re-validated according to GMP- and GLP-grade (good laboratory practices) methodologies before entering clinical testing.

The need for innovation, design, and GMP

Most pre-clinical trials using exosomes in cardiovascular regeneration used direct intracoronary or intramyocardial injections of exosomes after infarction.[12,14,15] Especially in the setting of remodelling after infarction, a process that can take up to 4 weeks, the expected biological effect of exosomes lasts about 24–48 h.[30] Studies have shown that in cell therapy the paracrine effect is limited due to low retention and survival of transplanted cells.[31]

Exosomes may have the potential to overcome the primary capillary bed after intravenous deliveries because of their size. Designed targeted exosomes could be administered continuously to further attenuate the effect of myocardial fibrosis after infarction. Especially in cancer therapeutics, researchers have designed exosomes that specifically target tumour cells by viral and non-viral modifications.[32] While viral approaches aimed at modifying the parent cell to produce exosomes with desired targeting surface proteins, non-viral approaches include all alterations made to the exosomes after isolation (*Figure 1*). There are examples for exosome modifications to improve the cardioprotective capacity of exosomes or engineer targeted exosomes.[33,34]

All of these studies have been limited to rodent models of AMI. Upscaling these methods for large animals can be challenging and translation into clinical practice requires compliance with increasingly stricter regulatory hurdles. In cancer research, study groups have gained some experience in the development of designed GMP-grade exosomes in larger quantities.

As an example, Mendt *et al.* have designed exosomes from bone marrow-derived MSCs (BM-MSCs) that deliver siRNAs to pancreatic cancer cells to inhibit oncogenic Kras.[18] During the development phase, native and designed exosomes were functionally and phenotypically characterized. In addition, the source of exosomes was evaluated by comparing exosome isolates from two different parent cells (BM-MSCs and BJ-fibroblasts). They discovered for instance, that MSCs in contrast to BJ-fibroblasts express CD47. CD47, a tetraspanin that can prevent microspinocytosis of exosomes by circulating monocytes and therefore reduces elimination of exosomes from the blood stream.[35] This study demonstrates that the development and evaluation of exosomes may be labour intensive but necessary to ensure successful clinical translation.

Conclusion

The limited data we have on the cardioprotective properties of exosomes suggests that they are a potential alternative to cell-based

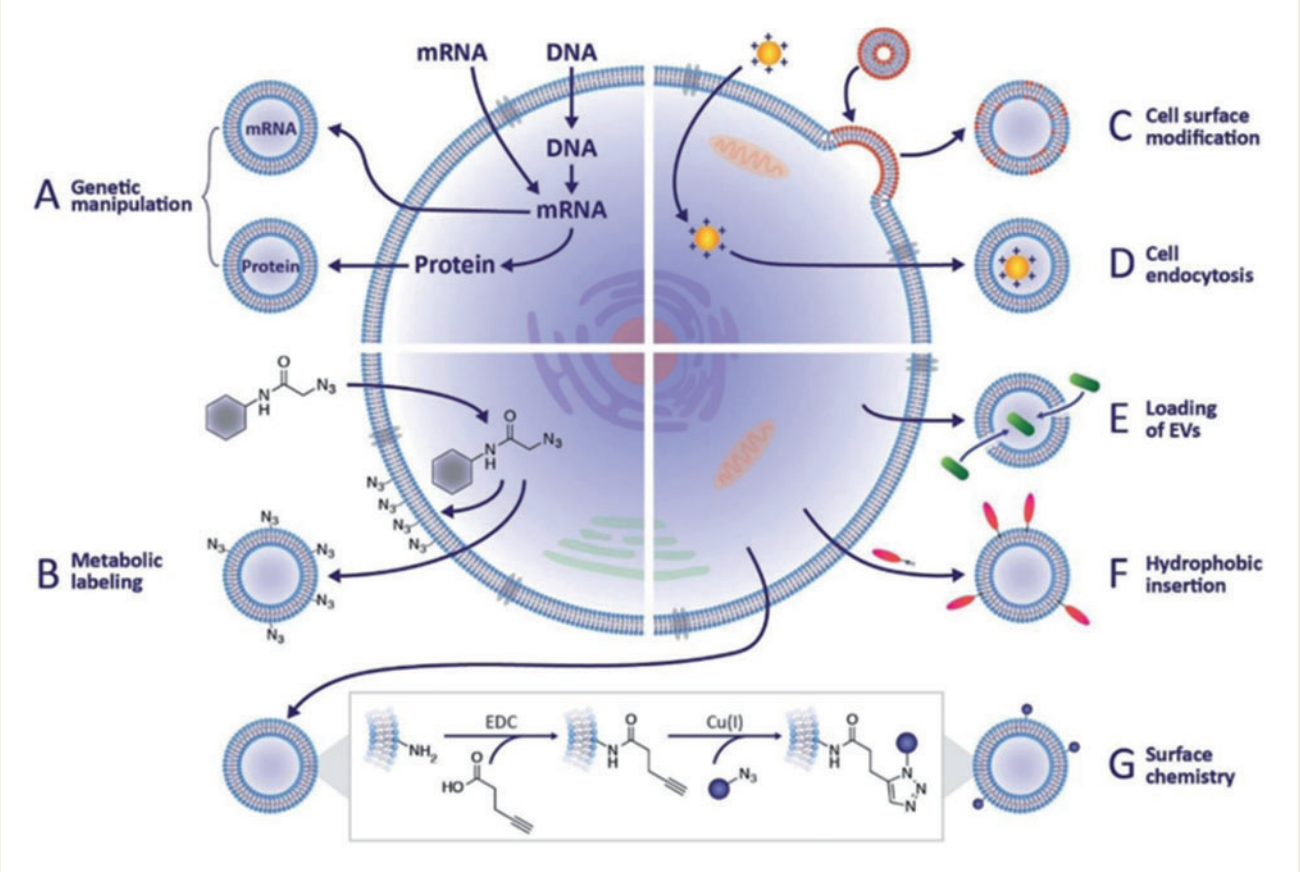

Figure 1 Engineering exosomes. Exosomes modification are achieved by either modification of the parent cell or by post-isolation modification. This includes hydrophobic insertion of targeting peptides or tumour associated anti-genes as well as electroporation, sonication, or lipotransfection. It has been also shown that overexpression of miRNAs and surface membrane proteins results in the production of designed exosomes. Reprinted with permission from Armstrong *et al.*[32]

therapies. It is however imperative that researchers in this field comply with recent consensus statements to prevent the push of immature therapeutic concepts into clinical trials. In addition, it will be crucial to further investigate the MoA of exosomes, improve *in vitro* screening methods, and develop isolation protocols that meet the high standards of GMP-grade production.

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