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Review

Bioreactors in tissue engineering: Advances in stem cell culture and three-dimensional tissue constructs

Bioreactors play an increasing role in tissue engineering—the generation of mammalian tissue equivalents in vitro. They can be applied for effective (stem) cell expansion, which is a crucial step concerning the fabrication of tissue engineering constructs of clinically relevant dimensions for which large cell numbers are needed. Furthermore, bioreactors are necessary for the maintenance of three-dimensional (3D) tissue engineering constructs during cultivation ex vivo, and can be used to further stimulate the cells with a variety of physical cues. The development of novel bioreactor systems as well as mathematical modeling of their characteristics is a fast-developing field of research. The present review provides a concise overview about recent developments in bioreactors for stem cell expansion, perfusion bioreactor systems for 3D cultures, and bioreactors for physical stimulation.

Keywords: Bioreactor / Cell culture / Physical stimulation / Perfusion / Scaffold

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1 Introduction

Tissue engineering (TE)—the creation of tissues of higher organisms ex vivo—is an emerging field of research. Possible applications for TE constructs are manifold. They comprise model systems of different complexity for basic research, tissue, and organ models for drug screening and safety evaluation up to artificial tissues for regenerative therapies in humans [1]. TE constructs, e.g. for the treatment of articular cartilage defects, have also been developed for the treatment of selected animals like horses. Commonly, TE includes the cultivation of mammalian cells on three-dimensional (3D) biomaterial scaffolds and therefore in most cases requires 3D cell culture technologies. Whereas TE at its beginning was not much more than the combination of one type of differentiated cells with a synthetic biomaterial scaffold ex vivo [2] nowadays complex tissue equivalents that may consist of two or even more different cell species and elaborated scaffolds, mimicking the extracellular matrix (ECM) of the respective tissue, can be generated and cultivated in the laboratory over weeks. Nevertheless, only few TE applications have found their way toward clinical application that is mostly due to legal hurdles and the high costs of TE-based therapies compared to conventional ones. But research in this field is developing fast and also safety issues are addressed and therefore TE approaches definitely will be utilised in future, at least for diseases or tissue defects that cannot be properly healed with conventional therapies.

In TE, bioreactors come into play for different purposes: (1) for cultivation/proliferation of cells (mostly in suspension) before seeding onto the scaffold material; (2) to maintain vitality of cells seeded on/in porous 3D scaffolds during cultivation, or (3) to achieve specific physical stimulation of the TE construct, supporting, e. g. cell/tissue differentiation, or to validate its function [3–5]. While the first mentioned application of bioreactors is described only in few cases for TE applications up to now, the cultivation of 3D TE constructs in bioreactor systems has become common during the last ca. 10 years. Above a distinct scaffold size limit—defined by several factors like cell type and proliferation rate, scaffold geometry, and porosity—cells cannot survive under static culture conditions in the centre of the construct because of a lack of oxygen and nutrients. In these cases dynamic cell culture is mandatory. Concerning the third field of application, mentioned above, it becomes more and more obvious that a variety of physical cues can strongly influence cellular behavior like cell morphology, proliferation, differentiation, matrix production, and protein expression in general. Therefore bioreactors that supply (mostly along with media perfusion) mechanical, electrical, or even magnetic cues are under intensive investigation. For distinct TE constructs, developed as implants for medical application, bioreactors are also necessary for validation of their functional capability. This is of great importance especially for artificial tissues that have to operate

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Abbreviations: ECM, extracellular matrix; **ESC**, embryonic stem cell; **GMP**, good manufacturing practice; **h**, human; **iPSC**, induced pluripotent stem cell; **MSC**, mesenchymal stem cell; **TE**, tissue engineering

in the blood flow like heart valves or artery segments that must properly act as valve or withstand the blood pressure directly after being implanted in the human body. For such applications, special bioreactors with a pulsatile media flow and adjustable pressure conditions have been developed, mimicking the conditions in the human heart or circulation. Cultivating TE constructs in such devices can be used for conditioning as well as for a final validation of quality and proper functioning.

Currently, an increasingly confusing array of systems for dynamic culture of TE constructs (with or without additional physical stimulation) is commercially available and furthermore many groups use self-made systems that complicates the comparability and reproducibility of the published results. Nevertheless, the present review tries to give a brief summary of state-of-the-art and the main research perspectives in the field of bioreactors for TE.

An additional field of application will not be described here but should at least be mentioned: the utilization of bioreactors for the production of substances that can be applied for TE. These could be growth factors (which means proteins) [6], used for the stimulation of cell proliferation or induction of cell differentiation—or biopolymers like polyhydroxybutyrate from which scaffolds for cell seeding can be manufactured [7]. For these purposes mostly nonmammalian cells like yeast or bacteria are used that require other cultivation conditions and therefore demand a very different bioreactor design.

2 Bioreactors for stem cell expansion

By definition, TE is limited to tissues that consist of adherent cells. Therefore research dedicated to nonadherent blood cells and respective models are not classified as TE. But two approaches concerning suspension cultivation of cells have drawn interest for TE applications. On the one hand, embryonic stem cells (ESC) that can differentiate in all types of cells and therefore are called pluripotent can be cultivated effectively in suspension culture–with or without the utilization of microcarriers (microbeads) to which the cells can adhere to. On the other hand, even mesenchymal stem cells (MSC) that are the progenitors of all mesenchymal tissues and therefore play the most important role in TE can be proliferated in suspension even without microbeads. Therefore the development of respective bioreactor systems has become an important field of research [8]. A third cell type gaining more and more interest in TE are the induced pluripotent stem cells (iPSC): pluripotent cells that can be derived from differentiated cells like dermal fibroblasts by reprogramming. The conditions for cultivating iPSC in suspension bioreactors are rather similar to those developed for ESC and therefore will not be described separately in this review.

2.1 Cultivation of embryonic and induced pluripotent stem cells in suspension bioreactors

ESC and iPSC (in the following combined under the term PSC) are fascinating cell types because of their ability to differentiate in principle in all cell types and generate even complex tissues or whole organs [9, 10]. They are an attractive source for research in many disciplines including TE but it remains still unclear whether PSC also can be utilized for therapies in humans. Actually only very few clinical trials are conducted with small numbers of patients and therefore no clear conclusions can be drawn yet concerning their effectiveness and safety [11, 12]. In addition, in most countries severe ethical concerns exist against the generation and utilisation of human ESC, accompanied by strict legal regulations. Nevertheless, many studies on improved and more effective culture conditions for expansion of PSC from different sources (mostly from mice, rats, and humans) have been published.

The main goals for improved PSC expansion technologies are listed in Table 1 that also demonstrates that bioreactors can contribute to most of the demands. Especially upscaling concerning the cell numbers is impossible with conventional static culture flasks. For effective PSC cultivation in most studies stirred-suspension bioreactors have been used and it could be demonstrated that pluripotency of the stem cells can be preserved even over long culture periods [8, 13, 14]. Beside stirred-suspension bioreactors in some studies also roller bottles have been used successfully [15]. Two main approaches have to be distinguished: in one case the cells are suspended directly in the cell culture medium and remain in a nonadherent state over the whole period of cultivation. ESC tend to form cell aggregates under such conditions called embroid bodies that influences the proliferation rate as well as the differentiation behavior [14]. In contrast, cells can be cultivated on so-called microcarriers that are small polymer or glass beads with diameters in the range of ca. 100–250 μm to which the cells can adhere to during suspension cultivation [14, 16]. Chen and coworkers recently have reviewed this technology and described the variety of microcarriers already available for this application, but also for cultivation of MSC [16]. Beside the type of bioreactor and choice of microcarrier (if applied) many other factors strongly influence PSC behavior during cultivation. Of special importance is the composition of the medium because animal-derived components like bovine serum may not be used if a clinical application is intended. It took many years to develop suitable xeno-free media that effectively support cell proliferation and maintenance of their pluripotency that recently was reviewed by Fan et al. [17]. Additional important aspects are the size of the cell aggregates during suspension culture, the hydrodynamic conditions (controlled by the bioreactor design and the agitation rate) and the oxygen concentration [18]. Many groups worldwide are currently trying to optimize the conditions for stirred suspension culture of PSC with remarkable success. For example, Krawetz and coworkers have developed a protocol leading to a 25-fold expansion of hESC over a cultivation period of only 6 days [19]. Therefore large-scale expansion of human PSC has become reality that is one of the prerequisites of a potential clinical application of these cells.

2.2 Expansion of mesenchymal stem cells using bioreactors

MSC are adherent cells and their capability to adhere to cell culture plastic is the most common way to isolate MSC, especially when derived from bone marrow that mostly consists of

Table 1. Main goals for expansion culture of PSC [13–15]

Aim	Motivation	Achievable with a bioreactor?
Simple setup	Reproducibility	No
Cheap instrumentation	Cost effectiveness	No
High proliferation rates	Cost effectiveness and time requirement	Yes
Preservation of pluripotency	Necessary for most applications	Yes
Ability for scale-up	Large cell numbers needed for e.g. clinical applications	Yes
Online monitoring and control	Reproducibility and effectiveness	Yes
Automation	Safety	Yes
Operable under GMP conditions	Necessary for most, e.g. clinical applications	Yes

GMP, good manufacturing practice.

nonadherent blood-related cells. The common method for expansion of MSC is still static cultivation in T-flasks that works well for small cell numbers and the utilization of those cells for research purposes. Disadvantage of this technique is that it is time and material consuming (and therefore expensive) to scale it up for the production of large cell numbers. In addition, it is difficult to perform the manual cell expansion under good manufacturing practice (GMP) conditions (which is mandatory in case of clinical applications) and a high variability concerning cell numbers and properties between the single flasks is often observed. Therefore many efforts have been made to develop automated culture conditions that can be easily scaled up and are cost effective and safe [8].

In contrast to the methods described so far a few studies exist about true suspension culture of MSC without any type of microcarriers. Baksh, Zandstra, and Davies from the University of Toronto demonstrated that human MSC derived from bone marrow can be effectively expanded in coculture with hematopoietic stem cells in stirred bioreactors [20]. They have published a protocol describing the culture conditions in detail and have proven the preservation of the multipotent properties of the cells [21], whereas continuous passaging under conventional static conditions is known to lead to progressive loss of "stemness." Similar to the findings for PSC it could be demonstrated that by expanding hMSC in stirred bioreactors significantly higher cell numbers can be achieved in contrast to conventional static cultures in which the cells adhere commonly to a flat plastic surface. Other groups have shown that similar protocols can be applied for hMSC of different origin, e.g. for placenta-derived MSC [22].

As already mentioned above, stirred-suspension bioreactor cultivation of MSC can also be performed by applying microcarriers to which the cells can adhere. Many groups have investigated this technology and could demonstrate that an effective upscaling is possible. State-of-the-art of microcarrier technology was recently reviewed by Chen et al. [16]. By further improving the cultivation conditions, especially cell attachment to the microcarriers, Yuan and coworkers could achieve a thousand fold increase of hMSC cell number over a cultivation period of 30 days compared to an only 200-fold increase in case of static culture [23]. Another approach has been described by Papadimitropoulos et al. [24]. They have expanded hMSC growing on/in porous 3D hydroxyapatite scaffolds in a commercially available perfusion bioreactor system and showed higher proliferation rates and a better preservation of the differentiation capacity compared to cells cultivated under standard 2D conditions [24]. The technology for large-scale production of hMSC has been reviewed by Jung et al. in 2012 [25].

3 Perfusion bioreactor systems for 3D TE constructs

The most important role of bioreactors in the field of TE is to keep 3D tissue constructs alive, to support further cell proliferation and allow differentiation toward the expected lineage. In the organism, tissues are supplied with oxygen and nutrients by the vascular system. Depending on the type of tissue, the density of blood capillaries is very high so that the distance between a cell and the nearest capillary is not bigger than a few hundreds of micrometres. This last distance is bridged by diffusion. An exception is articular cartilage that is the only mammalian tissue without a vascular system in which the cells (chondrocytes) therefore are only supplied by diffusion. The diffusion length is in addition strongly controlled by the composition and density of the ECM. Similarly, the morphology and especially porosity of a TE scaffold influences the effective diffusion length under cell culture conditions. Therefore it is impossible to define a general size limit up to which cells can be cultivated under static standard conditions; instead, this has to be figured out experimentally for each type of scaffold [26]. In addition, it should be considered that the pore size and interconnectivity could be altered during cultivation by the proliferating cells themselves and by matrix components, synthesized, and secreted by the cells. Also the cell type, proliferation rate as well as the differentiation status influence the needed amount of oxygen and nutrients. Finally, even the oxygen saturation of the medium can influence the cell differentiation itself.

Diffusion length can furthermore be influenced when cell cultivation is combined with mechanical stimulation of the cell-seeded TE construct. Cyclic compression as an example can strongly facilitate the supply of the cells by inducing fluid flow inside the scaffold. A similar mechanism is also postulated for articular cartilage in which part of the liquid, entrapped by the insoluble ECM components, is continuously squeezed out during movement of the joints and replaced by "fresh" liquid from the synovial fluid. Continuous mechanical stimulation of a TE construct therefore can be an alternative for perfusion culture (see Section 4).

Small TE constructs with a size of up to ca. 1 cm^3, suitable for basic research or as model systems are usually successfully

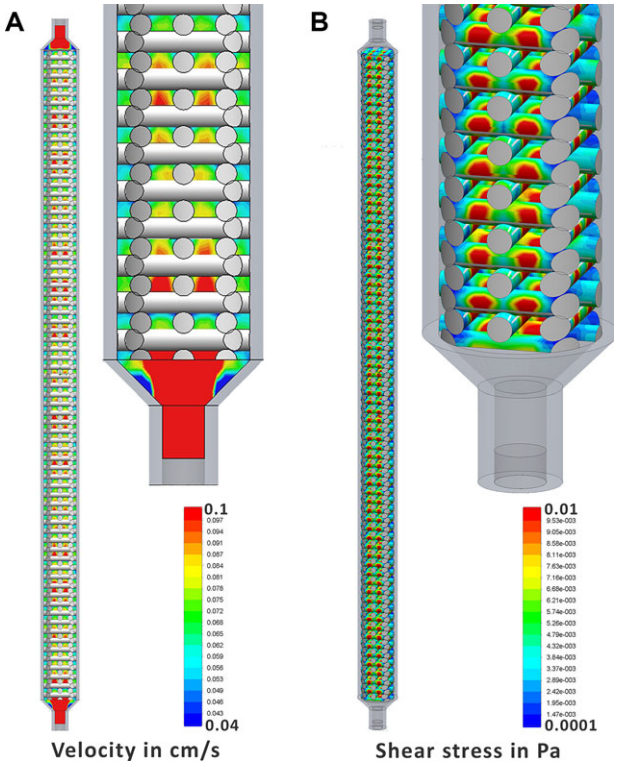

Figure 1. Simulation of (A) velocity and (B) shear stress to show the impact of scaffold length. Velocity at the inlet was set to 1 mL/min. Velocity levels and sheer stress levels are color-coded and refer to legends below the magnification of the inlet. The scaffold model was built in a rectangular pile wood structure consisting of 0.8 mm cylindrical strands with 0.8 mm spacing. CAD design was performed with Solidworks 2014 (Dassault Systèmes SolidWorks Corp.; Haar, Germany) and simulation was carried out with the simulation module of Solidworks 2014.

cultivated under static conditions [27]. In contrast, scaffold dimensions needed in the clinic for treatment of tissue defects in humans that cannot be healed with conventional therapies definitely require a dynamic cultivation step in a suitable bioreactor. In the following paragraph therefore the strategies for upscaling of TE constructs will be discussed more in detail, mostly based on studies performed in the field of bone TE. Finally, it should at least be mentioned that perfusion bioreactors can also be utilized for cell seeding of porous 3D scaffolds that could enhance the seeding efficacy and homogeneity of cell distribution [28].

3.1 Scale-up of perfusion bioreactor systems for 3D TE constructs

Especially when upscaling the size of a TE construct, static culture conditions including frequent exchange of culture medium may not be sufficient for survival of the cells in the centre of the scaffold resulting in central necrotic zones [29, 30]. Because of the ability to control fluid flow throughout the scaffold perfusion culture is the method of choice especially for cultivation of large TE constructs. The scaffold dimensions used currently in perfusion-based studies mainly focus on comparatively small sizes ranging from 0.04 to 2.7 cm^3 [31]. Higher volumes of 4.8 and even 10 cm^3 have been accomplished by the use of smaller scaffolds in a packed bed bioreactor [32] or especially adapted scaffold geometries [33]. As the majority of the perfusion approaches, these studies only address short distances of flow through the scaffolds [34–40]. This length in flow direction is an important parameter for perfusion-based systems in large scale, since it has impact on nutrition, stimulation by shear stress, and distribution of the cells. There is an unmet need of research to understand the impact of design changes in the up-scaled perfusion process and the underlying mechanisms [3, 29, 41, 42].

Furthermore it is necessary to adapt the bioreactor design, the cell number (i.e. cell density), the amount of medium and the flow rate, when changing the dimensions of the scaffold.

Hence the optimal parameter combination is the first step toward a successful up-scaling of perfusion culture [43, 44]. A common approach is the variation and analysis of one factor at a time in which several parameters like reactor design [31, 41, 45, 46], scaffold morphology [47–49], biomaterial composition [50–54], flow rate [31, 41, 49, 55], and shear stress [29, 41, 56, 57] have been frequently examined and reviewed. Unfortunately, such type of studies yields only a general operation range or vague tendency toward an optimum of the whole parameter set. Additionally, a lag of standardization and the variety of bioreactor systems in use is a very important issue in this context. For instance different flow rates have been frequently reviewed without adopting them to the diameter of the utilised scaffolds [42]. This may lead to biased results when comparing studies with different-sized scaffolds. The optimal parameters could be easily determined, if this broad array of studies would be accomplished by the convergence of experimental protocols and the introduction of relative sizes or dimensionless quantity.

The main goal should be to understand the cellular microenvironment that is called niche in case of stem cells. A very convenient approach to identify suitable parameters is the use of gradient cultures. Rupprecht et al. designed for example a tapered chamber generating different flow rates that allow the study of different shear stress values in one run [57].

Another promising strategy is the generation of windows of operation instead of examining one factor at a time [44]. By performing experiments with two independent parameters in a certain range with a defined threshold for one or more output variables, it is possible to narrow down the optimal parameter settings. Furthermore, the impact of the most promising parameters and their interaction to one another can be characterized by a quality of design technique utilizing a factorial design for experimental planning and evaluation [58–60]. Although, an elaborated experimental setup is necessary for defining the window of operation and the quality of design, both strategies represent a directed and efficient approach to mimic the cellular microenvironment.

In parallel with the experimental determination of parameters the computer simulation is an important tool to estimate and conceive process parameters especially in up-scaled perfusion culture. Several studies have been performed investigating the structure [61, 62], the cell distribution during seeding [63] and the sheer stress [40, 64–66]. To highlight the above-mentioned length in flow direction simulations of a 100 mm long porous

structure have been conducted. Figure 1A illustrates that the velocity within the construct is indeed changed with the height of the scaffold, which is reflected in periodically occurring velocity peaks. In contrast, the sheer stress shown in Fig. 1B renders no spatial differences on a macroscopic scale. The magnifications of the inlet reveal a pronounced variation of velocity and sheer stress in a local scale. This emphasizes that local and global changes of the perfused TE construct have to be measured and analyzed.

For the scale up the establishment of methods of bioprocess engineering within TE should be promoted for a further understanding of tissue development and regulation of the underlying processes, respectively for a better control of tissue formation ex vivo. Such methods may be the online sensing [67], inline process control and model-based growth kinetics [61,68]. Some of them are well established within suspension culture bioreactors and have to be translated to adherent culture conditions but some are basically different to already existing methods, due to the close cell-material interaction and the adherent nature of cells in TE. Finally, the long-term goal for the scale up is the translation toward a clinical application. This makes sterility, GMP compliance and easy handling mandatory and therewith imposes additional requirements toward the reactor design.

4 Bioreactors for physical stimulation

It is well known that not only biological and chemical properties of the local environment strongly influence the behavior of cells but also a variety of physical cues. Such cues can be "passive" like the stiffness of the matrix the cells adhere to [69] or "active" like mechanical, electrical, or magnetic stimulation. These physical cues can affect all aspects of cell function like adhesion, morphology, migration, differentiation, proliferation, and protein expression (of matrix proteins as well as enzymes that degrade matrix components etc.) and therefore might be utilized in TE for many purposes. For example, it could be demonstrated that MSC differentiation is strongly influenced by the stiffness of the microenvironment as well as active mechanical stimulation [70].

All cells in the living organism are exposed to mechanical forces, at least to the internal body pressure. But the cells of musculoskeletal tissues (bone, articular cartilage, and spinal discs, tendons, ligaments, and muscle tissue) encounter stronger forces than those of all other tissues. Therefore it is obvious that application of mechanical stimulation has been investigated up to now mostly for musculoskeletal TE [71]. Numerous studies have been published in the last ca. 20 years and just as many different instruments have been introduced. This is caused by the fact that the stimulation mode is strongly influenced by the properties of the scaffold material used and therefore for every TE construct a suitable technology has to be developed. Many studies have been performed with simple static cell culture devices like well plates to which the equipment for, e.g. mechanical stimulation has been attached. Partly, such instruments are also called *bioreactor* but the combination of perfusion culture systems and physical stimulation devices has become more common with time for which this term is more suitable. It is obvious that medium perfusion alone can lead to shear stress and therefore every type of perfusion or even only agitated culture conditions (e.g. in spinner flask or rotating wall bioreactors) will result in mechanical forces, present at the cell membranes. Table 2 summarizes the most common mechanical stimulation modes used in TE and for which type of tissue they are typically applied.

In a living organism not only mechanical forces are present, acting on cells and tissues, but also electrical fields. Live is based on the generation of potential differences, created by the cells of the organism with multiple ion pumps. Therefore electrical forces can strongly affect cellular behavior and should be utilizable for TE purposes too. Balint and coworkers recently have reviewed the approaches for applying electrical stimulation in TE and demonstrated the variety of bioreactor set-ups already developed for this purpose [82]. In most of the devices the generation of the electrical field is accompanied by the occurrence of a magnetic field which complicates a clear correlation between physical stimulus and cellular reaction. Therefore a new approach, published by Hess et al. should be highlighted in which pure electrical field stimulation could be realized in a cell culture device by transformer-like coupling [83].

Also the field of physical stimulation in TE is very fast developing that makes it difficult to give a concise overview. The variety of bioreactor systems developed so far for the utilization of physical stimulation in TE has been described very recently by a Korean group in a new review paper [84] and was delineated in several other publications in detail [3–5, 29, 31, 41–43, 73].

As already mentioned in the introduction bioreactors play a special role in TE of grafts that shall function in the circulation. Here, bioreactors are not only applied for stimulation of the cells during ex vivo cultivation of the constructs to achieve better, more tissue-like properties but also for validation of their functionality. Especially for artificial heart valves or artery segments a maximal reliability is crucial for a possible clinical application. For these special applications individual types of bioreactors have been developed, consisting of a pulsatile medium flow and hydrostatic pressure that mimics the conditions of the circulation [85, 86].

5 Concluding remarks

Bioreactors are nowadays one of the main components of TE. They become more and more common for the generation of large cell numbers and are advantageous for the maintenance of 3D TE constructs during cultivation ex vivo. Combined with devices for physical stimulation of cells bioreactors have evolved from instruments that have the only function to provide oxygen and nutrients to the cells and therefore keep the TE constructs alive to components that can in addition stimulate the cells by means of mechanical forces or electrical fields. With such stimulation the quality of TE constructs and its similarity to native tissues can be improved that increases the chance that TE will find its way to clinical application.

Table 2. Most common mechanical stimulation modes in TE and type of tissues

Stimulation mode	Type of tissue (most commonly applied for)	References
Cyclic compression	Bone, articular cartilage, and spinal disc	[72–75]
Cyclic elongation	Tendon and ligament	[76–78]
Torsion	Tendon and ligament	[79]
Shear stress	Bone, articular cartilage	[56, 75, 80]
Hydrostatic pressure	Articular cartilage	[74, 75]
Ultrasound	Bone	[81]
Pulsatile shear stress and hydrostatic pressure	Heart valves, artery segments	[85, 86]

Practical application

Tissue engineering has become an independent research discipline in the last few decades, with applications in basic research (in vitro models of tissues and organs), testing of new pharmaceutical agents and, most importantly, regenerative therapies for humans. Tissue engineering constructs of relevant dimensions are typically generated and maintained ex vivo in bioreactor systems. Therefore, the development of bioreactors designed specifically for the needs of tissue engineering is of great practical importance.

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6 References

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