Exploring dry storage as an alternative biobanking strategy for wildlife conservation, inspired by Nature

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Abstract

Close to 25% of species in the animal kingdom face the risk of extinction. A variety of stresses, largely anthropogenic in nature, and lack of sufficient resources and political support to counteract this biodiversity crisis, suggest that we will witness the extinction of many thousands of species in the coming years. Unless we act now to, at least, preserve samples from each and every threatened species, these extinct species will be lost forever. To preserve record of the world's biodiversity, establishment of genome resource banks is thus desirable. Presently, biobanking is done almost exclusively by cryopreservation, followed by maintenance of the samples under liquid nitrogen. Cryopreservation has satisfactory efficiency but it comes with a host of problems. The process is also highly species-specific. Like in many other walks of life, we can search Nature for better alternatives. When long-term preservation is desired, Nature opted for controlled drying rather than in water preservation via freezing. Nature also created an assortment of materials that protect organisms from damages during the drying and rehydration processes. Once dry, these organisms can survive extended periods of time and be resistant to extreme environmental stressors. Over the past 70 years researchers attempted applying this idea to preserved desiccation-sensitive mammalian cells in the dry form. Desiccation is experiencing increased interest in recent years with some exciting developments. Presented here an overview of dry preservation and its possible applications towards establishment of dry biobanks as part of our endeavour to preserve the world's biodiversity.

Keywords: Desiccation; Lyophilisation; Freeze-Drying; Endangered Species; Assisted Reproductive Technologies.

1. Introduction

The renowned Harvard biology professor Edward O. Wilson once said about ants, “We need them to survive, but they don’t need us at all.” He also said, “If all mankind were to disappear, the world would regenerate back to the rich state of equilibrium that existed ten thousand years ago. But if insects were to vanish, the environment would collapse into chaos.”

Species extinction is not new. It has been happening throughout the history of life on Earth. If one looks through the geologic record, probably over 95% of all species that have ever lived on this planet went extinct. Since speciation takes place in parallel to the process of extinction, the planet has not been depleted of its life forms (yet). Based on paleontological data, the rate of this ongoing extinction, which has been termed the background extinction rate or pre-human extinction rate, was estimated for many years at the extinction of one species out of every one million species per year (1 E/MSY) [1,2]. With an estimated 15 million species on this planet, this means that, on average, one can expect to witness the extinction of 15 species every year. Recently this estimate has been upended and the revised value is close to 0.1 E/MSY or, on average, the extinction of approximately 1.5 species per year [3]. This may be divided into species with restricted ranges for which extinction rate might be higher and those with widespread ranges for which it is considerably lower [1]. The planet has also witnessed five mass extinction events; events during which at least 75% of the estimated species present at the time went extinct over relatively short geologic time. These ‘Big Five’ events include the Late Ordovician event (~447-443 million years ago or MYA; estimated 86% of species lost), the Devonian event (~388-359 MYA; estimated 75% of species lost), the Late Permian event (~254-251 MYA; estimated 96% of species lost), the Late Triassic event (~208-200 MYA; estimated 80% of species lost), and the Late Cretaceous

event (~68-65 MYA; estimated 76% of species lost) [4]. The question that is now on the table is if we, humans, are bringing about the sixth mass extinction event and whether this event has already started [4-7]. If the current rate of species extinction already falls within the definition of a mass extinction event or not, there seem to be unanimous agreement that the current rate of extinction is orders of magnitude higher than the background rate, with some estimates putting it at as much as 10,000 times the background rate. Estimates, however, vary greatly, in part because we do not know exactly how many species there are out there, with some estimates going as high as 50 million. We also know very, very little about the status of the vast majority of these species. Only about 1.7 million species have been described and only about 4% of these have been assessed [8]. It is widely agreed that the leading cause for what we witness today are us, humans, and so, it is in our hands to make a change and attempt to fix things.

2. What can we do?

In recognition of this accelerated loss of biodiversity, representatives from almost all nations of the world gathered in 1992 in Rio de Janeiro, Brazil to reaffirm the 'Rio Declaration on Environment and Development' and to ratify the 'Convention on Biological Diversity' [9]. Since loss of biodiversity continued, the Parties to the Convention gathered again in 2002 and have "committed themselves to achieve by 2010 a significant reduction of the current rate of biodiversity loss at the global, regional and national levels as a contribution to poverty alleviation and to the benefit of all life on Earth" (https://www.cbd.int/2010-target). The year 2010 was also set by the United Nations as 'The International Year of Biodiversity'. However, with the approach of 2010, it became clear that not only the targets will not be achieved; even some of the tools to measure progress towards these targets were still missing [10]. With this knowledge, the Parties to the Convention gathered again in 2010 in Nagoya,

Japan and set forth new targets to be achieved by the year 2020 – the 'Aichi Biodiversity Targets' (https://www.cbd.int/sp/targets/). The United Nations has also set the current decade (2011-2020) as the 'Decade on Biodiversity'. Although progress has been made, it is clear by now that the Aichi Biodiversity Targets will not be achieved by 2020. For example, Target # 11 states: "By 2020, at least 17 per cent of terrestrial and inland water, and 10 per cent of coastal and marine areas, especially areas of particular importance for biodiversity and ecosystem services, are conserved through effectively and equitably managed, ecologically representative and well connected systems of protected areas and other effective area-based conservation measures, and integrated into the wider landscapes and seascapes." Recent studies suggest that we are still far from achieving these targets. At present 4.12% of the oceans, 10.2% of coastal marine areas under national jurisdiction and 14.7% of terrestrial and inland water areas are designated as protected areas [11]. A recent study, evaluating marine protected areas based on nine threshold levels for effective and equitable management, found that only 2% of the 433 marine protected areas analysed meet all nine thresholds, 8% failed to meet any of the thresholds, and only 21% met more than half of the nine thresholds [12]. And on land, a study published recently suggests that one third of the terrestrial protected areas are under sever human pressure, casting doubt as to the level of their effectiveness [13]. This leaves much room for improvement.

Sitting and waiting for things to happen is not the solution. Way too many species will go extinct before we find the ways to effectively protect the ecosystems in which they live. Some thousands years ago, according to the Bible, Noah built an ark and took a male and a female from each species so as to prevent their extinction during the big flood [14]. With the knowledge we have today, it is clear that with just one female and one male, most species would have gone extinct anyway or, if somehow survived, they would suffer from severe inbreeding. So Noah's solution is probably not a viable one. The alternative is to keep, under

human care, large enough populations from each species with sufficient genetic variability. Minimum viable population size ranges between several hundreds and several thousands, depending on the species and its distribution. With the limited resources (land, manpower, funds) and knowledge (reproductive biology, captive rearing and breeding), this option is also not a viable one for many species. Some years ago, the 'Global Seed Vault" was established on the island of Svalbard in Norway [15]. At present, seeds of almost 900,000 varieties of food plants are stored in this underground seed bank. The seeds are maintained at -18ºC with limited access to oxygen to ensure low metabolic activity and longevity. A similar approach, termed Genetic (or Genome) Resource Banks (GRBs), has also been suggested for wildlife conservation [16-19]. Animal cells and tissues, however, cannot survive and maintain their biological activity at -18ºC. Normally, much lower temperatures, in the range of liquid nitrogen temperature (-196ºC), are required for long-term preservation. Activities in this direction took place in many places around the world. At present there is no global GRB but rather consortia of organisations, research institutions, museums, zoological gardens and such, where various samples are stored (e.g. www.frozenark.org or www.amphibianark.org). There is also limited information available as to which samples are stored at which institution so efforts to preserve sufficient representative samples from each species are practically hindered by lack of information. When species-specific cryopreservation protocols have been devised, they are often of adequate efficiency. By very rough estimate, to date attempts to cryopreserve germplasm in mammals cover spermatozoa from approximately 115 species (~2% of all mammals), embryos from 51 species (~0.9% of all mammals) and oocytes from just a handful of species [20,21]. Births of live offspring from cryopreserved sperm were reported in about a fifth of these species and from cryopreserved embryos in about half of these species. The vast majority of the mammalian species in which cryopreservation has been attempted and resulted in live offspring, however, are domestic species, lab species, and

141 species of research and/or commercial value. Very few of them are truly wildlife species. A
142 number of causes stand behind this rather poor state of affairs. Species, by definition, are
143 different from each other and as such, their germplasm respond differently to the
144 cryopreservation process [22]. To overcome this, customising the protocols for each species
145 is necessary. As access to samples from wildlife species is limited, especially from those that
146 need it the most – the critically endangered species, progress has been limited and very, very
147 slow. Knowledge about the majority of species is still rather scarce. In the absence of
148 sufficient understanding of the anatomy, physiology, and reproductive biology of each
149 species, progress is expected to remain very slow. Add to this factors such as the risk
150 associated with the often needed anaesthesia or sedation, the distance of the animals from a
151 proper laboratory, and the general poor reproductive health of many individuals, and the
152 picture is clear – germplasm cryopreservation from wildlife (mammalian) species is destined
153 to see very limited progress in coming years. Furthermore, cryopreservation of germplasm
154 and tissues, and their maintenance under liquid nitrogen (LN), while effective, comes with
155 many inherent problems. It is expensive, energy-dependent, requires dedicated facilities and
156 trained personnel, continuous monitoring and uninterrupted LN and power supply, thus
157 putting such biobanks out of reach for many developing world countries and small businesses.
158 LN is also dangerous, complicates sample transport, may act as pathogen transmission
159 medium, and its production, transport and maintenance have high carbon footprint. A cheaper,
160 simpler and environmentally friendlier alternative is thus highly desirable.
161

162 **3. Nature's alternative**

163 When searching for an alternative, we would do right to turn to Nature and see how long-
164 term preservation has evolved there. A look around tells us that although some freezing
165 instances do exist [23], by far and large long-term preservation in Nature is rather done by

removing the water and thus bringing molecular interactions and metabolism to practical standstill. We know, however, that water is essential for life. Humans die when they lose over about 12% of their body water [24]. The wood frog, *Rana sylvatica*, can tolerate loss of as much as 50-60% of total body water before it dies [25]. The alpine cockroach, *Celatoblatta quinquemaculata*, from New Zealand, possibly a record holder, can lose as much as 82% of its body water before it expires [26]. But do we really have to die? A broad range of taxa, from bacteria and yeasts to complex organisms such as seeds, small trees, shrimp, fish embryos, and insects evolved mechanisms that allow complete desiccation without loss of viability, known as anhydrobiosis [27-29]. Even more striking, in the dry state, these organisms are biostable for decades/centuries, surviving extreme temperatures, pressures, and radiation [30-37]. Plants and animals are different in this respect. In plants, desiccation tolerance is usually in specialised tissues such as the seeds and pollen forms, but whole plants, known as the 'resurrection plants', are also known to be desiccation-tolerant [38]. Desiccation-tolerant animals, on the other hand, are mostly microscopic in size if the whole animal dehydrates, or they dehydrate at their larval stage. But size is not the only reason why these organisms can tolerate extreme desiccation. They usually live in places where water availability is ephemeral. As water evaporates and dry conditions set in, desiccation tolerant organisms start generating a variety of protective agents, collectively termed xeroprotectants, which they accumulate in and around the cells of their body. These xeroprotectants include: (i) very high concentration of sugars, mostly disaccharides such as trehalose or sucrose [39,40]; (ii) Late Embryogenesis Abundant proteins (LEAp) – a group of highly hydrophilic, intrinsically disordered, proteins that change their conformation based on their hydration status, and provide protection to specifically targeted cellular compartments [29]; (iii) a hydrophilic protein called anhydrin [41]; (iv) heat shock proteins [32]; (v) a variety of antioxidants [42], and more. Accumulation of these xeroprotectants is slow and gradual,

taking place in parallel to the drying process. Research into anhydrobiosis dates back more than 300 years to the microscopyst Antony van Leeuwenhoek, who reported about his discovery of anhydrobiotic 'animalcules', presumably a species of bdeloid rotifer (*Philodina roseola*) [43], in 1702 [44], and continued ever since [for historical overview see 45]. In the age of biomimicry (innovation inspired by nature), there is no reason why not to try and apply the knowledge gained from anhydrobiosis for the preservation of biological samples in the dry state.

4. The drying industry

Freeze-drying (lyophilisation), the more commonly used technique for drying biological samples, was known for hundreds of years as a method for meat and vegetable preservation. It has been in extensive use by people who lived in very high altitudes, like amongst the Andes mountain dwellers of South America. In more recent times, freeze-drying is used for preparation of food products such as instant coffee, tea and soup, fish food and even ice cream for NASA astronauts. It is also widely used for preparation of pharmaceuticals, viral, bacterial, fungal, or yeast products in a dry and convenient form for handling, transporting and long-term storage. To achieve these, while preserving the desired properties of the products or, in the case of bacteria, viruses, and yeast, its viability, the process of drying has been modelled [46,47] and a vast body of research took place, primarily in the food and pharmaceutical industries. Regrettably, there is very little interaction between these industries and the people involved in cells and tissue drying research, and so, little of the knowledge in the first has twinkled to the second. There is no doubt in our mind that interdisciplinary research is essential for this biobanking strategy to be pushed forward.

Arising from the knowledge accumulated from anhydrobiotic organisms, much of the research on stabilising cellular membranes and proteins concentrated on disaccharides,

primarily trehalose and sucrose. It was thought that these sugars help preserve cell membranes [48-50] and proteins [51] through two processes – glass transition and hydrogen bonds. Glass transition, or vitrification, is the process in which fluid practically solidifies without forming crystals. The glass transition temperature (Tg) of trehalose, for example, is high (115ºC) and as such it provides stability at relatively high supra-zero temperatures [52-54]. Trehalose is also unique in the fact that it does not completely lose its glass state even if it was slightly rehydrated. But vitrification alone is not enough to protect biological samples during drying [55]. It has thus been suggested that these sugars also provide structural stability by forming a large number of hydrogen bonds with membranes and proteins and by replacing water molecules during the drying process [55,56]. The drawback of these sugars, from the preservation perspective, is that they do not readily enter the cells. Anhydrobiotic organisms naturally possess the needed mechanisms to produce these sugars and load and unload them to and from the cells of their body. Desiccation-intolerant mammalian cells lack these mechanisms so ways to load and unload them are needed. One approach is to create a modified trehalose that is membrane permeable [57]. The alternative approach is to temporarily make the membrane permeable to this sugar by a variety of mechanisms that will be discussed later. Aside from trehalose (or sucrose), occasional use of LEA proteins, and minor modifications to the xeroprotective solutions, little has been reported in an attempt to discover the needed breakthrough in mammalian cells and gametes desiccation.

In the pharmaceutical industry, for example, the freeze-drying process is divided into two stages – primary drying and secondary drying. During primary drying the frozen water is sublimated at low temperatures and low pressure. During secondary drying, the unfrozen water is removed and thus the process can be done at supra-zero temperatures and higher pressure. Both stages have been studies extensively [46,58-60]. Such division is important for the food and pharmaceutical industries as by increasing the temperature and pressure during

241 the secondary drying stage they can save considerable amount of money on such industrial
242 process. Leaving the temperature and pressure low, as they were during the primary drying
243 stage, would prolong the process but otherwise the end result should be the same.
244 Determining the point at which to shift from primary to secondary drying and the point at
245 which the samples are dry enough (around 5% water per dry weight) is a tricky and difficult
246 endeavour. One option is to design the freeze-drying equipment in a way that would allow
247 removal of a sample for evaluation without affecting the rest or the process. Alternatively, the
248 use of the non-invasive Tunable Diode Laser Absorption Spectroscopy was suggested [60],
249 as it allows measuring water content in the product without removing it from the freeze-
250 drying equipment. In the absence of such sophisticated equipment, the alternative would be to
251 proceed by the trial-and-error approach.
252 The other issue that needs to be addressed is the rehydration stage, or imbibition – the act of
253 absorbing water and swelling. For many years, in the cryobiology field, research had
254 concentrated on the process of freezing the cells. This was basically so until Mazur and
255 colleagues reported on the importance of the warming velocity of the frozen samples [61,62].
256 In the drying arena we might be witnessing a similar process. Most studies in the field
257 concentrated on the drying media, the drying process and occasionally also the freezing
258 process. In both the scientific literature and the popular media, the approach of "just add
259 water" was largely taken by most. In principle, there is logic to this approach. During drying,
260 one removes the water while the solutes and cells remain behind, so what needs to be
261 replaced is the missing water. This approach, while devoid of side effects in case of dry
262 foodstuff, however, might be problematic and damaging to the cells. The sudden exposure to
263 pure water may result in rapid movement of the water into the cells, resulting in faster than
264 desired re-expansion and possibly cells' rupture in the process. To avoid this, a more gradual
265 rehydration process is desired. When vitrifying oocytes or embryos, for example, the cells are

exposed step-wise to increasing concentrations of the vitrification solution before vitrification, and vice versa during warming [20]. It is possible that a similar approach would turn beneficial when drying cells. Alternatively, as has been reported for bacteria, yeast, and pollen, rehydration with water can be done gradually by placing the samples for the initial rehydration stage in a humidity chamber or similar [28,63,64]. Another aspect of rehydration that has been discussed is the rehydration temperature. The temperature, once the cells get rehydrated, would affect the phase transition of the membrane and thus its stability and permeability. It is thus believed that rehydration temperature would have optimal range, as was shown, for example, for pollen and yeast [28,64,65]. How all these aspects will integrate and how different would this integration be when mammalian cells are concerned is still an open question.

5. Drying spermatozoa

Gametes, cells, and tissues of higher organisms are intolerant to desiccation and expire when drying exceeds certain threshold. We thus need to provide them with adequate protection during the drying and rehydration processes. If we could apply the idea of anhydrobiosis to biobanking and maintain the samples at ambient temperatures, we could save much in storage costs, space and logistics, make sample transportation simple and cheap, make biobanking an accessible technologies to the developing countries and small businesses where liquid nitrogen and/or power supply are unreliable or storage under liquid nitrogen is not practical. Dried samples will also weigh much less than their hydrated form, further reducing costs. And, unlike frozen samples, in the dried form samples can be used in small fractions as and when needed without damaging the rest. The process even may turn out to be a universal protocol applicable for all species; something that cryopreservation is not. It will also turn

biobanking into a safe green industry with low carbon footprint. So advantages are many and the research community has naturally showed interest in this alternative.

Probably the first reported attempt to apply drying to mammalian cell was the partial drying performed by Polge, Smith and Parkes [66]. In that study they diluted 1 mL of fowl semen with 1 mL of 20-30% glycerol in Ringer's solution, froze it to -79ºC, warmed it back to -25ºC and then dried it under vacuum for three hours. The samples "had the appearance of being dry." The glycerol and about 10% of the water remained with the sperm at the end of the drying process. The samples were rehydrated immediately and motile spermatozoa were seen in each microscopic field with the best being as much as 50% recovery. Waiting a couple of hours before rehydration resulted in zero recovery. It is clear that only partial drying took place here but this study set the stage for this ever-expanding field of inquiry. In the following years researchers concentrated on spermatozoa for obvious reasons – these cells are small, their DNA is less vulnerable to damages as it is highly condensed thanks to the protamines, and they contain very little water. During the 1950's and 1960's several reports on freeze-drying mammalian spermatozoa showed up in the scientific literature, including bovine [67-69] and human [70,71] spermatozoa. Some reports claimed to have obtained motile spermatozoa [72] and even offspring [73], but these successes could not be reproduced [67,74], not even by their own authors [75]. The usual outcome of sperm drying studies was that the spermatozoa were immotile and with damaged cytoplasmic membrane, so a way to get them into the oocyte was needed if fertilisation was desired. Sperm injection into oocytes was attempted at least since the 1960's [76] but 30 years had to pass till this technique, known at intracytoplasmic sperm injection (ICSI), was used to prove that non-motile spermatozoa can lead to embryonic development, pregnancy and in live offspring [77]. Attempts were made at injecting freeze-dried spermatozoa into oocytes before [78,79] but the final proof came from Wakayama and Yanagimachi who used freeze-dried mouse

315 spermatozoa to demonstrate that “dead” spermatozoon does not mean dead DNA, and that
316 freeze-dried spermatozoa are capable of directing normal embryonic development and
317 generating normal offspring following embryo transfer [80]. Their success started a new era
318 in dry preservation of sperm with activity taking place in a large number of (mostly) domestic
319 and lab animal species (Table 1). Live offspring have been reported in the mouse [80], rat
320 [81], rabbit [82], hamster [83], and horse [84].
321 A look through the published scientific literature in the field reviels an interesting picture.
322 While studies were conducted on a range of species, mice and cattle is by far the most
323 represented species and, for mice only, the species in which the largest number of studies
324 with offspring were reported (Table 1, Figure 1). Although the first report on successful
325 freeze drying was on epididymal mouse spermatozoa [80], the majority of studies performed
326 on larger mammals (rabbit, pig, cattle, sheep, horse) were performed on ejaculated
327 spermatozoa. Ejaculated and epididymal spermatozoa are different in their maturation status
328 and in their exposure to external factors. In a number of studies, performed on humans, it was
329 recurrently demonstrated that DNA quality was better in testicular spermatozoa compared to
330 ejaculated spermatozoa from the same subject [85,86]. Similar observation were also noted in
331 other species [87]. So, it is clear that there is some level of deterioration along the way from
332 the testis out. The only studies to date performed on drying epididymal spermatozoa from
333 large mammals include two reports on cat spermatozoa [88,89], and a study on ram
334 spermatozoa [90] in which the importance of single-strand DNA breaks (in adition to double-
335 strand breaks) was demonstrated. While a number of studies have evaluated DNA integrity,
336 this seem to be the only one in which single-strand breaks were evaluated. Another point that
337 emerge from looking at the published studies is related to the drying technique. Freeze-drying
338 is by far the mostly used technique for drying spermatozoa and other cell types (Figure 2).
339 Researchers have also sporadically attempted a large number of other techniques, including

evaporative-driying [91], spin-drying [92], heat-drying [93], microwave-drying [89], air-drying [94], vacuum-drying [95], convective drying [96], and vitri-drying [97]. Such a wide rage of techiques usually characterises a field in development. Take for example the field of oocyte and embryo vitrification. There are over 20 different vitrification carriers reported on [20]. Once the field will stabilise and will gain a strong foothold, probably one or two of these devices will emerge as the leaders and gain control over the bulk of the activity in the field. Similar process is also expected to happen in the field of drying. It can be assumed that the technique in which a major breakthrough will be achieved (e.g. large number of viable cells or motile spermatozoa after rehydration) will become the leader in the field. Finally, review of the various attempts to freeze-dry spermatozoa, two general approaches are observed. In most freeze-drying studies, the spermatozoa were snap-frozen without any attempt to maintain their membrane integrity (so called 'viability') [e.g. 80,98,99] while in other studies an attempt to obtain viable cells was made by using proper cryopreservation techniques [e.g. 90,100]. Does this suggest that most researchers approach the field with the assumption that spermatozoa would not survive the process and that as long as the DNA remains relatively intact, these cells can be used in ICSI? If one starts with such an assumption, naturally one would not bother with proper cryopreservation approach and the result is that progress towards achieving the desired breakthrough and obtaining motile spermatozoa after rehydration would be slower than desired. On the positive side, recent years saw great increase in interest in the field (Figure3) and hope is that in will further the field and lead to new discoveries.

6. Drying other cell types

Spermatozoa are small, with little water content and highly condensed cromatin, making them good candidate for successful reversible drying. Other cell types are normally much

larger in volume, making the drying process more challanging. Initial attempts took one of two approaches. Some researchers opted for drying cells that lack nuclear DNA in them, such as red blood cells and platelets [101-106]. Following this approach, the aim was to maintain the integrity of the membranes and cellular organeles and proteins, and thus the biological activity of these cells. For such an approach, liposomes were often used as a model. Although some positive results have been achieved, and the popular media showed much interest in this approach, to the best of our knowledge to date no such procedure has reached clinical use or anywhere near it. The other approach aimed to confer nucleated cells with desiccation tolerance. To achieve this, one of two strategies was followed. In the one approach, the cells were transfected with genes/proteins that were, based on knowledge from anhydrobiotic organisms, assumed to confer protection [107-109]. The alternative approach aimed to upload the cells with protective agents through alterations in membrane activity or permeability. Such alterations include activation of pre-existing ion channels [110,111], use of electroporation [106], utilisation of membrane phase transition during chilling and freezing to enhance uptake [112,113,114, Saragusty 2015, unpublished results], or through the use of haemolysin [89]. As an alternative, cells were incubated for various durations of time with protective agents with the hope that at least some of these will enter the cells by natural diffusion or endocytosis. Results show that the first approach (transfection) is the more successful one, resulting in enhanced cell viability and functionality following rehydration. While these results are encouraging, these are mostly suitable as a proof-of-concept approach but eventually an alternative technique that will not alter the cells and will be reversible upon rehydration should be aimed for.

Bacteria and yeast are routinely dried as a way to preserve them and extend their shelf life. Upon rehydration and incubation, resumption of biological activity is observed. Considering the fact that these organisms multiply at relatively high rates, it is sufficient that a small

fraction of organisms will survive the entire process to multiply and resume activity after rehydration. During work on drying spermatozoa, cells with intact membrane were often observed [90, Saragusty, unpublished data] though in small numbers and were immotile, in the range of 1%. Spermatozoa, however do not multiply. Cells, such as stem cells, have two advantages that may turn them into more suitable entities for drying. These cells multiply naturally so basically it would be enough that just a few will survive the process to allow re-establishment of the propulation after rehydration. Furthermore, the natural ability of these cells to multiply increases their plasticity and this may enhance their desiccation tolerance. In a study on CD34+ umbilical cord blood mononuclear cells, it was shown that such cells were able to maintain their membrane integrity through the process and to form colonies in culture after rehydration [115]. Similarly, cord blood haematopoietic stem and progenitor cells were able to form colonies upon rehydration and culture [116].

Attempts at drying other nucleated cells without tinkering with their genome or membranes thus far did not result in the recovery of full biological functionality following rehydration. This, however, does not mean that the cells are useless. About a decade ago, Loi and colleagues showed that, just like in spermatozoa, in somatic cells too “dead” cell does not mean “dead” DNA [117]. In their study, freeze-dried sheep granulosa cells, maintained at room temperature, were able to direct embryonic development to the blastocyst stage through somatic cell nuclear transfer (SCNT). These results were later duplicated using lyophilised porcine foetal fibroblasts [118] and mouse embryonic stem cells and cumulus cells [119]. Drying attempts were also reported in other cell types such as mouse J774 macrophage cells [120], mouse 3T3 fibroblasts [96,114], sheep lymphocytes [121], and cat germinal vesicles [122]. As of this point in time, there is no report of pregnancies, carried to term or not, resulting from embryos produced by nuclear transfer with desiccated somatic cells.

7. Future of dry biobanking

Studies on both somatic cells and spermatozoa have repeatedly demonstrated that the DNA in desiccated cells remains largely intact through the drying-rehydration process, particularly with respect to double-strand breaks. Furthermore, it is well known that the oocyte has a number of DNA repair pathways that actively repair DNA damages upon fertilisation [123]. These pathways are activated when the oocyte is presented with damages to structural basic sites, single- and double strand DNA breaks, and/or alternative base analogues to the orthodox ATGC. Naturally, if the damages surpass certain threshold or if they are present at critical sites, the zygote will eventually progress towards apoptosis. When possible, however, the oocyte will activate relevant repair pathways to reach a stable DNA molecule. Even so, the repair may not be ideal and may lead to mutations or chromosome aberrations, and single-strand breaks may end up becoming double-strand breaks during DNA replication. Still, much can be expected form the oocyte as was clearly shown in a number of studies that demonstrated *in vitro* embryonic development from desiccated cells, some of which with extensive DNA damage [90]. With the ability to generate embryos from both desiccated spermatozoa and desiccated somatic cells, probably two niches in the biobanking industry would be the primary targets of cells' drying.

The first niche would probably be for biobanking of dried spermatozoa. This could happen in at least three different fields. First, ICSI is routinely used in human fertility clinics so using immotile dried spermatozoa should not be vastly different from using immobilised fresh or frozen-thawed spermatozoa. Second, in domestic and laboratory animals, when preservation of genetic material from valuable animals is desired, the industry would be willing to go into the expense and effort of using ICSI to generate embryos when needed. Third, in wildlife conservation, samples are often preserved with no specific time limit, as a backup or security for the future. Once the need will arise, the desired technologies (oocyte collection, ICSI,

440 embryo culture and embryo transfer) would need to be developed before such spermatozoa
441 could be used. Before any and all these biobanking directions could be implemented, studies
442 will have to be conducted to show that drying and long-term preservation do not compromise
443 the cells in any possible way. This includes the genetic and epigenetic aspects as well as
444 embryonic, foetal, and offspring development.
445 The second niche would probably be long-term somatic cells preservation. Biobanking is
446 expensive so, if at least some of the preserved material can be maintained at room
447 temperature, free of liquid nitrogen storage, much in costs can be saved. This can be highly
448 useful when funds are very limited (e.g. in wildlife conservation) or when there is a need to
449 preserve samples from a large number of individuals [e.g. lab or domestic animals or the in
450 large initiatives such as the Precision Medicine Initiative (PMI) launched by President
451 Obama in 2015 in which preservation of multiple samples over the lifetime of a million
452 people is envisioned]. In these cases at least some of the samples can be dried and maintained
453 at ambient temperatures. Once rehydrated, such samples can be used for somatic cell nuclear
454 transfer. The derived embryos can be transferred or they can be used as a source for
455 embryonic stem cells. These cells can be directed *in vitro* towards the germ cell line and
456 further towards *in vitro* gametogenesis as was demonstrated by Hayashi and colleagues
457 [124,125] and others. Such *in vitro*-produced gametes can then potentially be used for *in vitro*
458 fertilisation, with embryo culture and transfer to follow. At present, the process of complete
459 *in vitro* gametogenesis has been demonstrated only in mice. It can be reasonably assumed,
460 however that the basic mechanisms controlling gametogenesis in mammals are highly
461 conserved, so applying this technology to other species would probably follow in the short-
462 to-mid term. As in the previous niche, long-term studies would have to demonstrate that the
463 technology is safe and harmless before it can be widely applied.

If, on the other hand, a major breakthrough in cells drying will come through and spermatozoa or somatic cells will be able to maintain their viability and biological functionality through the drying process, a much wider application of the technology can be foreseen. At the moment there are no indications that we are anywhere near such an optimistic development and studies in the field continue to be performed largely on the basis of trial-and-error. But, such discovery, like many other discoveries, may come any time by chance success or at a moment of Eureka.

8. Conclusions

Drying as a preservation technique is ubiquitous in Nature. It is known in a range of taxa in both the animal and the plant kingdoms. Following the biomimicry approach and applying drying as a biobanking paradigm to preserve desiccation-sensitive cells and tissues has many advantages. Conferring desiccation tolerance on such intolerant cells is complicated and, to date, has largely followed a trial-and-error approach. Although our knowledge of and about the process has expanded considerably over the past 70 years, and some offspring have been produced from dried spermatozoa in a few species, the field is still developing and waiting for a major breakthrough. As yet, we do not know what is the best approach to drying. Natures does not use freeze-drying as its chosen drying approach, yet, as we saw (Figure 2), lyophilisation is by far the most used technique. Similarly we do not know how best to rehydrate the cells. Combination of both dehydration and rehydration will have to be tackled to find the optimal desiccation alternative. And, before all these, it seem we need to really decide what we aim for. Do we aim for intact DNA? Or do we want viable cells with full resumption of their biological activity? A breakthrough, that would make drying desiccation-sensitive cells truly reversible and enable such cells resume their biological activity, might come as a major discovery or as a gradual, stepwise, progress. What is certain is that the field

would benefit considerably if those involved in the attempts to dry desiccation-sensitive cells would join forces with experts in anhydrobiology, plant physiology, computer modelling, and, of course, the food and pharmaceutical industries. Combining the available knowledge from these diverse fields might help reach the moment of Eureka we are all waiting for.

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9. References

[1] Pimm SL, Russell GJ, Gittleman JL, Brooks TM. The future of biodiversity. Science 1995;269: 347-350.

[2] Reid WV, Miller KR. Keeping Options Alive: The scientific basis for the conservation of biodiversity. Washington, DC: World Resources Institute, 1989;128.

[3] De Vos JM, Joppa LN, Gittleman JL, Stephens PR, Pimm SL. Estimating the normal background rate of species extinction. Conserv Biol 2015;29: 452-462.

[4] Barnosky AD, Matzke N, Tomiya S, Wogan GOU, Swartz B, Quental TB, Marshall C, McGuire JL, Lindsey EL, Maguire KC, Mersey B, Ferrer EA. Has the Earth's sixth mass extinction already arrived? Nature 2011;471: 51-57.

[5] Wake DB, Vredenburg VT. Colloquium paper: are we in the midst of the sixth mass extinction? A view from the world of amphibians. Proc Nat Acad Sci USA 2008;105 Suppl 1: 11466-11473.

[6] Ceballos G, Ehrlich PR, Barnosky AD, García A, Pringle RM, Palmer TM. Accelerated modern human–induced species losses: Entering the sixth mass extinction. Sci Adv 2015;1: e1400253.

[7] Dirzo R, Young HS, Galetti M, Ceballos G, Isaac NJB, Collen B. Defaunation in the Anthropocene. Science 2014;345: 401-406.

[8] Monastersky R. Life - a status report. Nature 2014;516: 159-161.

[9] Convention on Biological Diversity. Convention on Biological Diversity. In: United Nations (ed), 1992;28.

[10] Walpole M, Almond REA, Besancon C, Butchart SHM, Campbell-Lendrum D, Carr GM, Collen B, Collette L, Davidson NC, Dulloo E, Fazel AM, Galloway JN, Gill M, Goverse T, Hockings M, Leaman DJ, Morgan DHW, Revenga C, Rickwood CJ, Schutyser F, Simons S, Stattersfield AJ, Tyrrell TD, Vie J-C, Zimsky M. Tracking

progress toward the 2010 biodiversity target and beyond. Science 2009;325: 1503-1504.

[11] UNEP-WCMC, IUCN. Protected Planet Report 2016. Cambridge, UK and Gland, Switzerland: UNEP-WCMC and IUCN, 2016.

[12] Gill DA, Mascia MB, Ahmadia GN, Glew L, Lester SE, Barnes M, Craigie I, Darling ES, Free CM, Geldmann J, Holst S, Jensen OP, White AT, Basurto X, Coad L, Gates RD, Guannel G, Mumby PJ, Thomas H, Whitmee S, Woodley S, Fox HE. Capacity shortfalls hinder the performance of marine protected areas globally. Nature 2017;543: 665.

[13] Jones KR, Venter O, Fuller RA, Allan JR, Maxwell SL, Negret PJ, Watson JEM. One-third of global protected land is under intense human pressure. Science 2018;360: 788-791.

[14] Genesis, The Bible, ~3500 BC?;Chapter 1 number 27.

[15] Charles D. A 'Forever' seed bank takes root in the arctic. Science 2006;312: 1730-1731.

[16] Wildt DE. Genetic resource banks for conserving wildlife species: justification, examples and becoming organized on a global basis. Anim Reprod Sci 1992;28: 247-257.

[17] Veprintsev BN, Rott NN. Conserving genetic resources of animal species. Nature 1979;280: 633-634.

[18] Holt WV, Bennett PM, Volobouev V, Watwon PF. Genetic resource banks in wildlife conservation. J Zool 1996;238: 531-544.

[19] Saragusty J. Genome Banking for Vertebrates Wildlife Conservation. In: Katkov II (ed), Current Frontiers in Cryobiology. Rijeka, Croatia: InTech, 2012;293-368.

[20] Saragusty J, Arav A. Current progress in oocyte and embryo cryopreservation by slow freezing and vitrification. Reproduction 2011;141: 1-19.

[21] Charlton SJ, Nielsen MB, Pedersen CR, Thomsen L, Kristjansen MP, Sørensen TB, Pertoldi C, Strand J. Strong heterogeneity in advances in cryopreservation techniques in the mammalian orders. Zool Sci 2018;35: 1-22.

[22] Thurston LM, Watson PF, Holt WV. Semen cryopreservation: a genetic explanation for species and individual variation? Cryo Lett 2002;23: 255-262.

[23] Shatilovich AV, Tchesunov AV, Neretina TV, Grabarnik IP, Gubin SV, Vishnivetskaya TA, Onstott TC, Rivkina EM. Viable nematodes from Late Pleistocene permafrost of the Kolyma river lowland. Doklady Biol Sci 2018;480: 100-102.

[24] Piantadosi CA. The Biology of Human Survival: Live and Death in Extreme Environments. New York, NY, USA: Oxford University Press, 2003;263.

[25] Churchill TA, Storey KB. Dehydration tolerance in wood frogs: a new perspective on development of amphibian freeze tolerance. Am J Physiol Regul Integr Comp Physiol 1993;265: R1324-R1332.

[26] Sinclair BJ. Water relations of the freeze-tolerant New Zealand alpine cockroach *Celatoblatta quinquemaculata* (Dictyoptera: Blattidae). J Insect Physiol 2000;46: 869-876.

[27] Loi P, Iuso D, Czernik M, Zacchini F, Ptak G. Towards storage of cells and gametes in dry form. Trends Biotech 2013;31: 688-695.

[28] Crowe JH, Hoekstra FA, Crowe LM. Anhydrobiosis. Annu Rev Physiol 1992;54: 579-599.

[29] Hand SC, Menze MA, Toner M, Boswell L, Moore D. LEA proteins during water stress: Not just for plants anymore. Annu Rev Physiol 2011;73: 115-134.

[30] Horikawa DD, Kunieda T, Abe W, Watanabe M, Nakahara Y, Yukuhiro F, Sakashita T, Hamada N, Wada S, Funayama T, Katagiri C, Kobayashi Y, Higashi S, Okuda T. Establishment of a rearing system of the extremotolerant tardigrade *Ramazzottius varieornatus*: A new model animal for astrobiology. Astrobiology 2008;8: 549-556.

[31] Ono F, Saigusa M, Uozumi T, Matsushima Y, Ikeda H, Saini NL, Yamashita M. Effect of high hydrostatic pressure on to life of the tiny animal tardigrade. J Phys Chem Solids 2008;69: 2297-2300.

[32] Ramløv H, Westh P. Cryptobiosis in the eutardigrade *Adorybiotus (Richtersius) coronifer*: Tolerance to alcohols, temperature and *de novo* protein synthesis. Zool Anz 2001;240: 517-523.

[33] Jönsson KI, Rabbow E, Schill RO, Harms-Ringdahl M, Rettberg P. Tardigrades survive exposure to space in low Earth orbit. Curr Biol 2008;18: R729-R731.

[34] Horikawa DD, Sakashita T, Katagiri C, Watanabe M, Kikawada T, Nakahara Y, Hamada N, Wada S, Funayama T, Higashi S, Kobayashi Y, Okuda T, Kuwabara M. Radiation tolerance in the tardigrade *Milnesium tardigradum*. Int J Rad Biol 2006;82: 843-848.

[35] Hengherr S, Worland MR, Reuner A, Brümmer F, Schill RO. High−temperature tolerance in anhydrobiotic tardigrades is limited by glass transition. Physiol Biochem Zool 2009;82: 749-755.

[36] Sallon S, Solowey E, Cohen Y, Korchinsky R, Egli M, Woodhatch I, Simchoni O, Kislev M. Germination, genetics, and growth of an ancient date seed. Science 2008;320: 1464.

[37] García A. Anhydrobiosis in bacteria: From physiology to applications. J Biosci 2011;36: 939-950.

[38] Scott P. Resurrection plants and the secrets of eternal leaf. Ann Bot 2000;85: 159-166.

[39] Crowe JH, Crowe LM, Chapman D. Preservation of membranes in anhydrobiotic organisms: The role of trehalose. Science 1984;223: 701-703.

[40] Leslie SB, Israeli E, Lighthart B, Crowe JH, Crowe LM. Trehalose and sucrose protect both membranes and proteins in intact bacteria during drying. Appl Environ Microbiol 1995;61: 3592-3597.

[41] Goyal K, Walton LJ, Browne JA, Burnell AM, Tunnacliffe A. Molecular anhydrobiology: Identifying molecules implicated in invertebrate anhydrobiosis. Integr Comp Biol 2005;45: 702-709.

[42] Cornette R, Kanamori Y, Watanabe M, Nakahara Y, Gusev O, Mitsumasu K, Kadono-Okuda K, Shimomura M, Mita K, Kikawada T, Okuda T. Identification of anhydrobiosis-related genes from an expressed sequence tag database in the cryptobiotic midge *Polypedilum vanderplanki* (Diptera; Chironomidae). J Biol Chem 2010;285: 35889-35899.

[43] Tunnacliffe A, Lapinski J. Resurrecting Van Leeuwenhoek's rotifers: A reappraisal of the role of disaccharides in anhydrobiosis. Phil Trans R Soc Lon B Biol Sci 2003;358: 1755-1771.

[44] van Leeuwenhoek A. On certain Animalcules found in the sediment in gutters on the roofs of houses, The Selected Works of Antony Van Leeuwenhoek. London: G. Sidney, 1800;207-214.

[45] Keilin D. The Leeuwenhoek Lecture - The problem of anabiosis or latent life: history and current concept. Proc R Soc Lon B Biol Sci 1959;150: 149-191.

[46] Mascarenhas WJ, Akay HU, Pikal MJ. A computational model for finite element analysis of the freeze-drying process. Comput Method Appl M 1997;148: 105-124.

623 [47] Velardi SA, Barresi AA. Development of simplified models for the freeze-drying
624 process and investigation of the optimal operating conditions. Chem Eng Res Design
625 2008;86: 9-22.
626 [48] Mouradian R, Womersley C, Crowe LM, Crowe JH. Preservation of functional
627 integrity during long term storage of a biological membrane. Biochim Biophys Acta
628 Biomemb 1984;778: 615-617.
629 [49] Sun WQ, Leopold AC, Crowe LM, Crowe JH. Stability of dry liposomes in sugar
630 glasses. Biophys J 1996;70: 1769-1776.
631 [50] Crowe LM, Crowe JH. Trehalose and dry dipalmitoylphosphatidylcholine revisited.
632 Biochim Biophys Acta Biomemb 1988;946: 193-201.
633 [51] Wang W. Lyophilization and development of solid protein pharmaceuticals. Int J
634 Pharm 2000;203: 1-60.
635 [52] Crowe JH, Carpenter JF, Crowe LM. The role of vitrification in anhydrobiosis. Annu
636 Rev Physiol 1998;60: 73-103.
637 [53] Cummins HZ, Zhang H, Oh J, Seo J-A, Kim HK, Hwang Y-H, Yang YS, Yu YS, Inn
638 Y. The liquid–glass transition in sugars: Relaxation dynamics in trehalose. J Non-
639 Cryst Solids 2006;352: 4464-4474.
640 [54] Reis J, Sitaula R, Bhowmick S. Water activity and glass transition temperatures of
641 disaccharide based buffers for desiccation preservation of biologics. J Biomed Sci
642 Eng 2009;8: 594-605.
643 [55] Crowe JH, Leslie SB, Crowe LM. Is vitrification sufficient to preserve liposomes
644 during freeze-drying? Cryobiology 1994;31: 355-366.
645 [56] Tsvetkova NM, Phillips BL, Crowe LM, Crowe JH, Risbud SH. Effect of sugars on
646 headgroup mobility in freeze-dried dipalmitoylphosphatidylcholine bilayers: solid-
647 state 31P NMR and FTIR studies. Biophys J 1998;75: 2947-2955.

[57] Abazari A, Meimetis LG, Budin G, Bale SS, Weissleder R, Toner M. Engineered trehalose permeable to mammalian cells. PLOS ONE 2015;10: e0130323.

[58] Pikal MJ. Use of laboratory data in freeze drying process design: Heat and mass transfer coefficients and the computer simulation of freeze drying. PDA J Pharm Sci Tech 1985;39: 115-139.

[59] Pikal MJ, Shah S, Roy ML, Putman R. The secondary drying stage of freeze drying: Drying kinetics as a function of temperature and chamber pressure. Int J Pharm 1990;60: 203-207.

[60] Schneid SC, Gieseler H, Kessler WJ, Luthra SA, Pikal MJ. Optimization of the secondary drying step in freeze drying using TDLAS technology. AAPS PharmSciTech 2011;12: 379-387.

[61] Seki S, Mazur P. Effect of warming rate on the survival of vitrified mouse oocytes and on the recrystallization of intracellular ice. Biol Reprod 2008;79: 727-737.

[62] Seki S, Mazur P. The dominance of warming rate over cooling rate in the survival of mouse oocytes subjected to a vitrification procedure. Cryobiology 2009;59: 75-82.

[63] Morgan CA, Herman N, White PA, Vesey G. Preservation of micro-organisms by drying; A review. J Microbiol Methods 2006;66: 183-193.

[64] Poirier I, Marechal PA, Richard S, Gervais P. *Saccharomyces cerevisiae* viability is strongly dependant on rehydration kinetics and the temperature of dried cells. J Appl Microbiol 1999;86: 87-92.

[65] Hoekstra FA. Imbibitional chilling injury in pollen: Involvement of the respiratory chain. Plant Physiol 1984;74: 815-821.

[66] Polge C, Smith AU, Parkes AS. Revival of spermatozoa after vitrification and dehydration at low temperatures. Nature 1949;164: 666.

672 [67] Saacke RG, Almquist JO. Freeze-drying of bovine spermatozoa. Nature 1961;192:
673 995-996.
674 [68] Sherman JK. Freezing and freeze-drying of bull spermatozoa. Am J Physiol 1957;190:
675 281-286.
676 [69] Bialy G, Smith VR. Freeze-drying of bovine spermatozoa. J Dairy Sci 1957;40: 739-
677 745.
678 [70] Sherman JK. Freezing and freeze-drying of human spermatozoa. Fertil Steril 1954;5:
679 357-371.
680 [71] Sherman JK. Improved methods of preservation of human spermatozoa by freezing
681 and freeze-drying. Fertil Steril 1963;14: 49-64.
682 [72] Meryman HT, Kafig E. Survival of spermatozoa following drying. Nature 1959;184:
683 470-471.
684 [73] Yushchenko NP. Proof of the possibility of preserving mammalian spermatozoa in a
685 dried state. Proc Lenin Acad Agric Sci 1957;22: 37-40.
686 [74] Nei T, Nagase H. Attempts to freeze-dry bull spermatozoa. Low Temp Sci B 1961;19:
687 107-115.
688 [75] Meryman HT, Kafig E. Special Article: Freeze-drying of bovine spermatozoa. J
689 Reprod Fertil 1963;5: 87-94.
690 [76] Hiramoto Y. Microinjection of the live spermatozoa into sea urchin eggs. Exp Cell
691 Res 1962;27: 416-426.
692 [77] Palermo G, Joris H, Devroey P, Van Steirteghem AC. Pregnancies after
693 intracytoplasmic injection of single spermatozoon into an oocyte. Lancet 1992;340:
694 17-18.

[78] Hoshi K, Yanagida K, Katayose H, Yazawa H. Pronuclear formation and cleavage of mammalian eggs after microsurgical injection of freeze-dried sperm nuclei. Zygote 1994;2: 237-242.

[79] Uehara T, Yanagimachi R. Microsurgical injection of spermatozoa into hamster eggs with subsequent transformation of sperm nuclei into male pronuclei. Biol Reprod 1976;15: 467-470.

[80] Wakayama T, Yanagimachi R. Development of normal mice from oocytes injected with freeze-dried spermatozoa. Nat Biotechnol 1998;16: 639-641.

[81] Kaneko T, Serikawa T. Successful long-term preservation of rat sperm by freeze-drying. PLOS ONE 2012;7: e35043.

[82] Liu JL, Kusakabe H, Chang CC, Suzuki H, Schmidt DW, Julian M, Pfeffer R, Bormann CL, Tian XC, Yanagimachi R, Yang X. Freeze-dried sperm fertilization leads to full-term development in rabbits. Biol Reprod 2004;70: 1776-1781.

[83] Muneto T, Horiuchi T. Full-term development of hamster embryos produced by injecting freeze-dried spermatozoa into oocytes. J Mamm Ova Res 2011;28: 32-39.

[84] Choi YH, Varner DD, Love CC, Hartman DL, Hinrichs K. Production of live foals via intracytoplasmic injection of lyophilized sperm and sperm extract in the horse. Reproduction 2011;142: 529-538.

[85] Greco E, Scarselli F, Iacobelli M, Rienzi L, Ubaldi F, Ferrero S, Franco G, Anniballo N, Mendoza C, Tesarik J. Efficient treatment of infertility due to sperm DNA damage by ICSI with testicular spermatozoa. Hum Reprod 2005;20: 226-230.

[86] Moskovtsev SI, Jarvi K, Mullen JBM, Cadesky KI, Hannam T, Lo KC. Testicular spermatozoa have statistically significantly lower DNA damage compared with ejaculated spermatozoa in patients with unsuccessful oral antioxidant treatment. Fertil Steril 2010;93: 1142-1146.

[87] Yamauchi Y, Ajduk A, Riel JM, Ward MA. Ejaculated and epididymal mouse spermatozoa are different in their susceptibility to nuclease-dependent DNA damage and in their nuclease activity. Biol Reprod 2007;77: 636-647.

[88] Ringleb J, Waurich R, Wibbelt G, Streich WJ, Jewgenow K. Prolonged storage of epididymal spermatozoa does not affect their capacity to fertilise *in vitro*-matured domestic cat (*Felis catus*) oocytes when using ICSI. Reprod Fertil Dev 2011;23: 818-825.

[89] Patrick JL, Elliott GD, Comizzoli P. Structural integrity and developmental potential of spermatozoa following microwave-assisted drying in the domestic cat model. Theriogenology 2017;103: 36-43.

[90] Palazzese L, Gosálvez J, Anzalone DA, Loi P, Saragusty J. DNA fragmentation in epididymal freeze-dried ram spermatozoa impairs embryo development. J Reprod Dev 2018;64: 393-400.

[91] Li MW, Biggers JD, Elmoazzen HY, Toner M, McGinnis L, Lloyd KCK. Long-term storage of mouse spermatozoa after evaporative drying. Reproduction 2007;133: 919-929.

[92] Chakraborty N, Chang A, Elmoazzen H, Menze M, Hand S, Toner M. A spin-drying technique for lyopreservation of mammalian cells. Ann Biomed Eng 2011;39: 1582-1591.

[93] Lee K-B, Niwa K. Fertilization and development *in vitro* of bovine oocytes following intracytoplasmic injection of heat-dried sperm heads. Biol Reprod 2006;74: 146-152.

[94] Moisan AE, Leibo SP, Lynn JW, Gómez MC, Pope CE, Dresser BL, Godke RA. Embryonic development of felid oocytes injected with freeze-dried or air-dried spermatozoa. Cryobiology 2005;51: 373-374 (abstract).

[95] Meyers SA, Li MW, Enders AC, Overstreet JW. Rhesus macaque blastocysts resulting from intracytoplasmic sperm injection of vacuum-dried spermatozoa. J Med Primatol 2009;38: 310-317.

[96] Acker JP, Fowler A, Lauman B, Cheley S, Toner M. Survival of desiccated mammalian cells: Beneficial effect of isotonic media. Cell Preserv Technol 2002;1: 129-140.

[97] Arav A. VitDrying of reproductive cells using the "Minimum drop size (MDS)" technique. Cryobiology 2013;67: 421 (Abstract).

[98] Kamada Y, Wakayama S, Shibasaki I, Ito D, Kamimura S, Ooga M, Wakayama T. Assessing the tolerance to room temperature and viability of freeze-dried mice spermatozoa over long-term storage at room temperature under vacuum. Sci Rep 2018;8: 10602.

[99] Domingo P, Olaciregui M, González N, De Blas I, Gil L. Long-term preservation of freeze-dried rabbit sperm by adding rosmarinic acid and different chelating agents. Cryobiology 2018;81: 174-177.

[100] Martins CF, Bao SN, Dode MN, Correa GA, Rumpf R. Effects of freeze-drying on cytology, ultrastructure, DNA fragmentation, and fertilizing ability of bovine sperm. Theriogenology 2007;67: 1307-1315.

[101] Goodrich RP, Sowemimo-Coker SO, Zerez CR, Tanaka KR. Preservation of metabolic activity in lyophilized human erythrocytes. Proc Nat Acad Sci USA 1992;89: 967-971.

[102] Arav A, Natan D. Freeze drying (lyophilization) of red blood cells. J Trauma Acute Care Surg 2011;70: S61-S64.

[103] Arav A, Natan D. Freeze drying of red blood cells: The use of directional freezing and a new radio frequency lyophilization device. Biopreserv Biobank 2012;10: 386-394.

[104] Crowe JH, Crowe LM, Wolkers WF, Oliver AE, Ma X, Auh J-H, Tang M, Zhu S, Norris J, Tablin F. Stabilization of dry mammalian cells: Lessons from nature. Integr Comp Biol 2005;45: 810-820.

[105] Wolkers WF, Tablin F, Crowe JH. From anhydrobiosis to freeze-drying of eukaryotic cells. Comp Biochem Physiol A Mol Integr Physiol 2002;131: 535-543.

[106] Zhou X, Yuan J, Liu J, Liu B. Loading trehalose into red blood cells by electroporation and its application in freeze-drying. Cryo Lett 2010;31: 147-156.

[107] Hand SC, Menze MA. Molecular approaches for improving desiccation tolerance: insights from the brine shrimp *Artemia franciscana*. Planta 2015;242: 379-388.

[108] Li S, Chakraborty N, Borcar A, Menze MA, Toner M, Hand SC. Late embryogenesis abundant proteins protect human hepatoma cells during acute desiccation. Proc Nat Acad Sci USA 2012;109: 20859-20864.

[109] Ma X, Jamil K, MacRae TH, Clegg JS, Russell JM, Villeneuve TS, Euloth M, Sun Y, Crowe JH, Tablin F, Oliver AE. A small stress protein acts synergistically with trehalose to confer desiccation tolerance on mammalian cells. Cryobiology 2005;51: 15-28.

[110] Elliott GD, Liu X-H, Cusick JL, Menze M, Vincent J, Witt T, Hand S, Toner M. Trehalose uptake through P2X7 purinergic channels provides dehydration protection. Cryobiology 2006;52: 114-127.

[111] Sitaula R, Elmoazzen H, Toner M, Bhowmick S. Desiccation tolerance in bovine sperm: A study of the effect of intracellular sugars and the supplemental roles of an antioxidant and a chelator. Cryobiology 2009;58: 322-330.

[112] Oldenhof H, Zhang M, Narten K, Bigalk J, Sydykov B, Wolkers WF, Sieme H. Freezing-induced uptake of disaccharides for preservation of chromatin in freeze-dried stallion sperm during accelerated aging. Biol Reprod 2017;97: 892-901.

794 [113] Wolkers W. Freeze-dried cells and tissues. Cryobiology 2013;67: 422 (Abstract).
795 [114] Zhang M, Oldenhof H, Sydykov B, Bigalk J, Sieme H, Wolkers WF. Freeze-drying of
796 mammalian cells using trehalose: Preservation of DNA integrity. Sci Rep 2017;7:
797 6198.
798 [115] Natan D, Nagler A, Arav A. Freeze-drying of mononuclear cells derived from
799 umbilical cord blood followed by colony formation. PLOS ONE 2009;4: e5240.
800 [116] Buchanan SS, Pyatt DW, Carpenter JF. Preservation of differentiation and clonogenic
801 potential of human hematopoietic stem and progenitor cells during lyophilization and
802 ambient storage. PLOS ONE 2010;5: e12518.
803 [117] Loi P, Matsukawa K, Ptak G, Clinton M, Fulka Jr. J, Natan Y, Arav A. Freeze-dried
804 somatic cells direct embryonic development after nuclear transfer. PLOS ONE 2008;3:
805 e2978.
806 [118] Das ZC, Gupta MK, Uhm SJ, Lee HT. Lyophilized somatic cells direct embryonic
807 development after whole cell intracytoplasmic injection into pig oocytes. Cryobiology
808 2010;61: 220-224.
809 [119] Ono T, Mizutani E, Li C, Wakayama T. Nuclear transfer preserves the nuclear
810 genome of freeze-dried mouse cells. J Reprod Dev 2008;54: 486-491.
811 [120] Chakraborty N, Biswas D, Parker W, Moyer P, Elliott GD. A role for microwave
812 processing in the dry preservation of mammalian cells. Biotechnol Bioeng 2008;100:
813 782-796.
814 [121] Iuso D, Czernik M, Di Egidio F, Sampino S, Zacchini F, Bochenek M, Smorag Z,
815 Modlinski JA, Ptak G, Loi P. Genomic stability of lyophilized sheep somatic cells
816 before and after nuclear transfer. PLOS ONE 2013;8: e51317.

817 [122] Graves-Herring JE, Wildt DE, Comizzoli P. Retention of structure and function of the
818 cat germinal vesicle after air-drying and storage at supra-zero temperature. Biol
819 Reprod 2013;88: 139, 131-137.
820 [123] Ménézo Y, Dale B, Cohen M. DNA damage and repair in human oocytes and
821 embryos: a review. Zygote 2010;18: 357-365.
822 [124] Hayashi K, Ogushi S, Kurimoto K, Shimamoto S, Ohta H, Saitou M. Offspring from
823 oocytes derived from in vitro primordial germ cell-like cells in mice. Science
824 2012;338: 971-975.
825 [125] Hayashi K, Ohta H, Kurimoto K, Aramaki S, Saitou M. Reconstitution of the mouse
826 germ cell specification pathway in culture by pluripotent stem cells. Cell 2011;146:
827 519-532.
828 [126] Kwon IK, Park KE, Niwa K. Activation, pronuclear formation, and development *in*
829 *vitro* of pig oocytes following intracytoplasmic injection of freeze-dried spermatozoa.
830 Biol Reprod 2004;71: 1430-1436.
831 [127] Men NT, Kikuchi K, Nakai M, Fukuda A, Tanihara F, Noguchi J, Kaneko H, Linh
832 NV, Nguyen BX, Nagai T, Tajima A. Effect of trehalose on DNA integrity of freeze-
833 dried boar sperm, fertilization, and embryo development after intracytoplasmic sperm
834 injection. Theriogenology 2013;80: 1033-1044.
835 [128] Meng X, Gu X, Wu C, Dai J, Zhang T, Xie Y, Wu Z, Liu L, Ma H, Zhang D. [Effect
836 of trehalose on the freeze-dried boar spermatozoa]. Sheng Wu Gong Cheng Xue Bao
837 2010;26: 1143-1149 (abstract).
838 [129] Nakai M, Kashiwazaki N, Takizawa A, Maedomari N, Ozawa M, Noguchi J, Kaneko
839 H, Shino M, Kikuchi K. Effects of chelating agents during freeze-drying of boar
840 spermatozoa on DNA fragmentation and on developmental ability *in vitro* and *in vivo*
841 after intracytoplasmic sperm head injection. Zygote 2007;15: 15-24.

842 [130] Olaciregui M, Luño V, González N, Domingo P, de Blas I, Gil L. Chelating agents in
843 combination with rosmarinic acid for boar sperm freeze-drying. Reprod Biol 2017;17:
844 193-198.
845 [131] Li X-X, Diao Y-F, Wei H-J, Wang S-Y, Cao X-Y, Zhang Y-F, Chang T, Li D-L, Kim
846 MK, Xu B. Tauroursodeoxycholic acid enhances the development of porcine embryos
847 derived from *in vitro*-matured oocytes and evaporatively dried spermatozoa. Sci Rep
848 2017;7: 6773.
849 [132] García A, Gil L, Malo C, Martínez F, Kershaw-Young C, de Blas I. Effect of different
850 disaccharides on the integrity and fertilising ability of freeze-dried boar spermatozoa:
851 A preliminary study. Cryo Lett 2014;35: 277-285.
852 [133] Men NT, Kikuchi K, Furusawa T, Dang-Nguyen TQ, Nakai M, Fukuda A, Noguchi J,
853 Kaneko H, Viet Linh N, Xuan Nguyen B, Tajima A. Expression of DNA repair genes
854 in porcine oocytes before and after fertilization by ICSI using freeze-dried sperm.
855 Anim Sci J 2016;87: 1325-1333.
856 [134] Pfaller W, Rovan E, Mairbäurl H. A comparison of the ultrastructure of spray-frozen
857 and freeze-etched or freeze-dried bull and boar spermatozoa with that after chemical
858 fixation. J Reprod Fertil 1976;48: 285-290.
859 [135] Shahba MI, El-Sheshtawy RI, El-Azab A-SI, Abdel-Ghaffar AE, Ziada MS, Zaky AA.
860 The effect of freeze-drying media and storage temperature on ultrastructure and DNA
861 of freeze-dried buffalo bull spermatozoa. Asia Pac J Reprod 2016;5: 524-535.
862 [136] Hara H, Tagiri M, Hirabayashi M, Hochi S. Effect of cake collapse on the integrity of
863 freeze-dried bull spermatozoa. Reprod Fertil Dev 2013;26: 144 (Abstract).
864 [137] Keskintepe L, Pacholczyk G, Machnicka A, Norris K, Curuk MA, Khan I, Brackett
865 BG. Bovine blastocyst development from oocytes injected with freeze-dried
866 spermatozoa. Biol Reprod 2002;67: 409-415.

[138] Hara H, Tagiri M, Hwang I-S, Takahashi M, Hirabayashi M, Hochi S. Adverse effect of cake collapse on the functional integrity of freeze-dried bull spermatozoa. Cryobiology 2014;68: 354-360.

[139] Abdalla H, Hirabayashi M, Hochi S. Demethylation dynamics of the paternal genome in pronuclear-stage bovine zygotes produced by *in vitro* fertilization and ooplasmic injection of freeze-thawed or freeze-dried spermatozoa. J Reprod Dev 2009;55: 433-439.

[140] Larson EV, Graham EF. Freeze-drying of spermatozoa. Dev Biol Stand 1976;36: 343-348.

[141] Hara H, Abdalla H, Morita H, Kuwayama M, Hirabayashi M, Hochi S. Procedure for bovine ICSI, not sperm freeze-drying, impairs the function of the microtubule-organizing center. J Reprod Dev 2011;57: 428-432.

[142] Abdalla H, Hirabayashi M, Hochi S. The ability of freeze-dried bull spermatozoa to induce calcium oscillations and resumption of meiosis. Theriogenology 2009;71: 543-552.

[143] Albright JL, Erb RE, Ehlers MH. Freeze-drying bovine spermatozoa. J Dairy Sci 1958;41: 206-207.

[144] Jeyendran RS, Graham EF, Schmehl MKL. Fertility of dehydrated bull semen. Cryobiology 1981;18: 292-300.

[145] Liu Q-C, Chen T-e, Huang X-Y, Sun F-Z. Mammalian freeze-dried sperm can maintain their calcium oscillation-inducing ability when microinjected into mouse eggs. Biochem Biophys Res Commun 2005;328: 824-830.

[146] Martins CF, Dode MN, Bao SN, Rumpf R. The use of the acridine orange test and the TUNEL assay to assess the integrity of freeze-dried bovine spermatozoa DNA. Genet Mol Res 2007;6: 94-104.

892 [147] Jeyendran RS, Hunter AG, Graham EF. Alteration of seminal proteins during freeze-
893 drying of bovine semen. J Dairy Sci 1983;66: 887-891.
894 [148] Sitaula R, Jimenez J, Bhowmick S. Osmotic damage as a predictor of motility loss
895 during convective desiccation of bovine sperm. Biopreserv Biobank 2013;11: 371-
896 378.
897 [149] Magalhães LCO, Melo-Oña CM, Sudano MJ, Paschoal DM, Crocomo LF,
898 Ackermann CL, Villaverde AISB, Landim-Alvarenga FC, Lopes MD. An easy-to-
899 perform method to assess viability of feline freeze-dried sperm. Reprod Fertil Dev
900 2012;25: 182 (abstract).
901 [150] Kaneko T, Ito H, Sakamoto H, Onuma M, Inoue-Murayama M. Sperm preservation
902 by freeze-drying for the conservation of wild animals. PLOS ONE 2014;9: e113381.
903 [151] Watanabe H, Asano T, Abe Y, Fukui Y, Suzuki H. Pronuclear formation of freeze-
904 dried canine spermatozoa microinjected into mouse oocytes. J Assist Reprod Genet
905 2009;26: 531-536.
906 [152] Olaciregui M, Luño V, Gonzalez N, De Blas I, Gil L. Freeze-dried dog sperm:
907 Dynamics of DNA integrity. Cryobiology 2015;71: 286-290.
908 [153] Czarny NA, Harris MS, De Iuliis GN, Rodger JC. Acrosomal integrity, viability, and
909 DNA damage of sperm from dasyurid marsupials after freezing or freeze drying.
910 Theriogenology 2009;72: 817-825.
911 [154] Katayose H, Matsuda J, Yanagimachi R. The ability of dehydrated hamster and
912 human sperm nuclei to develop into pronuclei. Biol Reprod 1992;47: 277-284.
913 [155] Meyers SA. Dry storage of sperm: applications in primates and domestic animals.
914 Reprod Fertil Dev 2006;18: 1-5.
915 [156] Alonso A, Baca Castex C, Ferrante A, Pinto M, Castañeira C, Trasorras V,
916 Gambarotta MC, Losinno L, Miragaya M. *In vitro* equine embryo production using

air-dried spermatozoa, with different activation protocols and culture systems. Andrologia 2015;47: 387-394.

[157] Olaciregui M, Luño V, Martí JI, Aramayona J, Gil L. Freeze-dried stallion spermatozoa: evaluation of two chelating agents and comparative analysis of three sperm DNA damage assays. Andrologia 2016;48: 900-906.

[158] Kusakabe H, Yanagimachi R, Kamiguchi Y. Mouse and human spermatozoa can be freeze-dried without damaging their chromosomes. Hum Reprod 2008;23: 233-239.

[159] Arav A, Saragusty J. Directional freezing of sperm and associated derived technologies. Anim Reprod Sci 2016;169: 6-13.

[160] Gianaroli L, Magli MC, Stanghellini I, Crippa A, Crivello AM, Pescatori ES, Ferraretti AP. DNA integrity is maintained after freeze-drying of human spermatozoa. Fertil Steril 2012;97: 1067-1073.

[161] McEvoy A, Roberts P, Yap K, Matson P. Development of a simplified method of human semen storage for the testing of sperm DNA fragmentation using the Halosperm G2 test kit. Fertil Steril 2014;102: 981-988.

[162] Zhu W-j, Li J, Xiao L-j. Changes on membrane integrity and ultrastructure of human sperm after freeze-drying. J Reprod Contracept 2016;27: 76-81.

[163] Kaneko T, Nakagata N. Relation between storage temperature and fertilizing ability of freeze-dried mouse spermatozoa. Comp Med 2005;55: 140-144.

[164] Kaneko T, Nakagata N. Improvement in the long-term stability of freeze-dried mouse spermatozoa by adding of a chelating agent. Cryobiology 2006;53: 279-282.

[165] Kaneko T, Serikawa T. Long-term preservation of freeze-dried mouse spermatozoa. Cryobiology 2012;64: 211-214.

[166] Ward MA, Kaneko T, Kusakabe H, Biggers JD, Whittingham DG, Yanagimachi R. Long-term preservation of mouse spermatozoa after freeze-drying and freezing without cryoprotection. Biol Reprod 2003;69: 2100-2108.

[167] Wakayama S, Kamada Y, Yamanaka K, Kohda T, Suzuki H, Shimazu T, Tada MN, Osada I, Nagamatsu A, Kamimura S, Nagatomo H, Mizutani E, Ishino F, Yano S, Wakayama T. Healthy offspring from freeze-dried mouse spermatozoa held on the International Space Station for 9 months. Proc Nat Acad Sci USA 2017;114: 5988-5993.

[168] Kaneko T. The latest improvements in the mouse sperm preservation. In: Lewandoski M (ed), Mouse Molecular Embryology: Methods and Protocols. Boston, MA: Springer US, 2014;357-365.

[169] Kaneko T. Simple sperm preservation by freeze-drying for conserving animal strains. In: Pruett-Miller SM (ed), Chromosomal Mutagenesis: Springer New York, 2015;317-329.

[170] Kaneko T, Whittingham DG, Overstreet JW, Yanagimachi R. Tolerance of the mouse sperm nuclei to freeze-drying depends on their disulfide status. Biol Reprod 2003;69: 1859-1862.

[171] Li M-W, Willis BJ, Griffey SM, Spearow JL, Lloyd KCK. Assessment of three generations of mice derived by ICSI using freeze-dried sperm. Zygote 2009;17: 239-251.

[172] Kusakabe H, Szczygiel MA, Whittingham DG, Yanagimachi R. Maintenance of genetic integrity in frozen and freeze-dried mouse spermatozoa. Proc Nat Acad Sci USA 2001;98: 13501-13506.

963 [173] Kaneko T, Whittingham DG, Yanagimachi R. Effect of pH value of freeze-drying
964 solution on the chromosome integrity and developmental ability of mouse
965 spermatozoa. Biol Reprod 2003;68: 136-139.
966 [174] Kawase Y, Tachibe T, Jishage K-i, Suzuki H. Transportation of freeze-dried mouse
967 spermatozoa under different preservation conditions. J Reprod Dev 2007;53: 1169-
968 1174.
969 [175] Bhowmick S, Zhu L, McGinnis L, Lawitts J, Nath BD, Toner M, Biggers J.
970 Desiccation tolerance of spermatozoa dried at ambient temperature: production of
971 fetal mice. Biol Reprod 2003;68: 1779-1786.
972 [176] Kawase Y, Araya H, Kamada N, Jishage K-i, Suzuki H. Possibility of long-term
973 preservation of freeze-dried mouse spermatozoa. Biol Reprod 2005;72: 568-573.
974 [177] Kawase Y, Hani T, Kamada N, Jishage K-i, Suzuki H. Effect of pressure at primary
975 drying of freeze-drying mouse sperm reproduction ability and preservation potential.
976 Reproduction 2007;133: 841-846.
977 [178] Kawase Y, Suzuki H. A study on freeze-drying as a method of preserving mouse
978 sperm. J Reprod Dev 2011;57: 176-182.
979 [179] Kusakabe H, Tateno H. Characterization of chromosomal damage accumulated in
980 freeze-dried mouse spermatozoa preserved under ambient and heat stress conditions.
981 Mutagenesis 2011;26: 447-453.
982 [180] Kusakabe H, Tateno H. Prevention of high-temperature-induced chromosome damage
983 in mouse spermatozoa freeze-dried using Ca2+ chelator-containing buffer alkalinized
984 with NaOH or KOH. Cryobiology 2017;79: 71-77.
985 [181] Kawase Y, Wada NA, Jishage K. Evaluation of DNA fragmentation of freeze-dried
986 mouse sperm using a modified sperm chromatin structure assay. Theriogenology
987 2009;72: 1047-1053.

988 [182] Kusakabe H. Chromosomal integrity and DNA damage in freeze−dried spermatozoa.
989 Reprod Med Biol 2011;10: 199-210.
990 [183] Kusakabe H, Kamiguchi Y. Chromosomal integrity of freeze-dried mouse
991 spermatozoa after 137Cs γ-ray irradiation. Mutat Res 2004;556: 163-168.
992 [184] McGinnis LK, Zhu L, Lawitts JA, Bhowmick S, Toner M, Biggers JD. Mouse sperm
993 desiccated and stored in trehalose medium without freezing. Biol Reprod 2005;73:
994 627-633.
995 [185] Elmoazzen HY, Lee GY, Li MW, McGinnis LK, Kent Lloyd KC, Toner M, Biggers
996 JD. Further optimization of mouse spermatozoa evaporative drying techniques.
997 Cryobiology 2009;59: 113-115.
998 [186] Biggers JD. Evaporative drying of mouse spermatozoa. Reprod Biomed Online
999 2009;19: 115-124.
1000 [187] Li M-W, Baridon B, Trainor A, Djan E, Koehne A, Griffey SM, Biggers JD, Toner M,
1001 Lloyd KCK. Mutant mice derived by ICSI of evaporatively dried spermatozoa exhibit
1002 expected phenotype. Reproduction 2012;143: 449-453.
1003 [188] Li M-W, Biggers JD, Toner M, Griffey SM, Lloyd KKC. Phenotypic analysis of
1004 C57BL/6J and FVB/NJ mice generated using evaporatively dried spermatozoa. Comp
1005 Med 2007;57: 469-475.
1006 [189] Liu J, Lee GY, Lawitts JA, Toner M, Biggers JD. Preservation of mouse sperm by
1007 convective drying and storing in 3-O-Methyl-D-Glucose. PLOS ONE 2012;7: e29924.
1008 [190] Liu J, Lee GY, Lawitts JA, Toner M, Biggers JD. Live pups from evaporatively dried
1009 mouse sperm stored at ambient temperature for up to 2 years. PLOS ONE 2014;9:
1010 e99809.

[191] Poleo GA, Godke RR, Tiersch TR. Intracytoplasmic sperm injection using cryopreserved, fixed, and freeze-dried sperm in eggs of Nile tilapia. Mar Biotechnol 2005;7: 104-111.

[192] Lee K-B, Park K-E, Kwon I-K, Tripurani SK, Kim KJ, Lee JH, Niwa K, Kim MK. Develop to term rat oocytes injected with heat-dried sperm heads. PLOS ONE 2013;8: e78260.

[193] Hirabayashi M, Kato M, Ito J, Hochi S. Viable rat offspring derived from oocytes intracytoplasmically injected with freeze-dried sperm heads. Zygote 2005;13: 79-85.

[194] Hochi S, Watanabe K, Kato M, Hirabayashi M. Live rats resulting from injection of oocytes with spermatozoa freeze-dried and stored for one year. Mol Reprod Dev 2008;75: 890-894.

[195] Kaneko T, Kimura S, Nakagata N. Importance of primary culture conditions for the development of rat ICSI embryos and long-term preservation of freeze-dried sperm. Cryobiology 2009;58: 293-297.

[196] Klooster KL, Burruel VR, Meyers SA. Loss of fertilization potential of desiccated rhesus macaque spermatozoa following prolonged storage. Cryobiology 2011;62: 161-166.

[197] Sánchez-Partida LG, Simerly CR, Ramalho-Santos J. Freeze-dried primate sperm retains early reproductive potential after intracytoplasmic sperm injection. Fertil Steril 2008;89: 742-745.

[198] Anzalone DA, Palazzese L, Iuso D, Martino G, Loi P. Freeze-dried spermatozoa: An alternative biobanking option for endangered species. Anim Reprod Sci 2018;190: 85-93.

[199] Arav A, Idda A, Nieddu SM, Natan Y, Ledda S. High post-thaw survival of ram sperm after partial freeze-drying. J Assist Reprod Genet 2018;In Press.

[200] Olaciregui M, Luño V, Domingo P, González N, Gil L. *In vitro* developmental ability of ovine oocytes following intracytoplasmic injection with freeze-dried spermatozoa. Sci Rep 2017;7: 1096.

1041 **Table 1**: sperm drying listed by species, drying technique and endpoint of the study.

Species	Drying technique	Study endpoint	Reference
Boar	Freeze-drying	Blastocyst formation	[126-130]
Boar	Evaporative-drying	Blastocyst formation	[131]
Boar	Freeze-drying	MPN	[132]
Boar	Freeze-drying	Oocyte gene activation	[133]
Boar	Freeze-drying	Ultrastructural analysis	[134]
Buffalo	Freeze-drying	DNA integrity	[135]
Bull	Freeze-drying	Blastocyst formation	[100,136-138]
Bull	Freeze-drying	Zygote, methylation	[139]
Bull	Freeze-drying	Fertilization[1]	[72,140]
Bull	Freeze-drying	Sperm asters and microtubule formation	[141]
Bull	Freeze-drying	Meiosis resumption	[142]
Bull	Freeze-drying	Sperm motility	[67-69,74,75,143,144]

[1] The authors (Meryman & Kafig) and others later failed to duplicate these results [67,74,75].

Species	Drying technique	Study endpoint	Reference
Bull	Freeze-drying	Ultrastructural analysis	[134]
Bull	Freeze-drying	DNA integrity	[136,145,146]
Bull	Freeze-drying	Seminal proteins integrity	[147]
Bull	Heat-drying	Blastocyst formation	[93]
Bull	Convective-drying	Sperm motility, membrane integrity	[111,148]
Cat	Freeze-drying	Blastocyst formation	[94]
Cat	Freeze-drying	Cleavage rate	[88]
Cat	Freeze-drying	DNA integrity	[149]
Cat	Air-drying	Blastocyst formation	[94]
Cat	Microwave-drying	Blastocyst formation	[89]
Chimpanzee	Freeze-drying	MPN development in mouse oocyte	[150]
Dog	Freeze-drying	MPN formation	[151]
Dog	Freeze-drying	DNA integrity	[152]
Fat-tailed dunnart	Freeze-drying	Motility, viability, acrosome and DNA integrity	[153]
Giraffe	Freeze-drying	MPN development in mouse oocyte	[150]

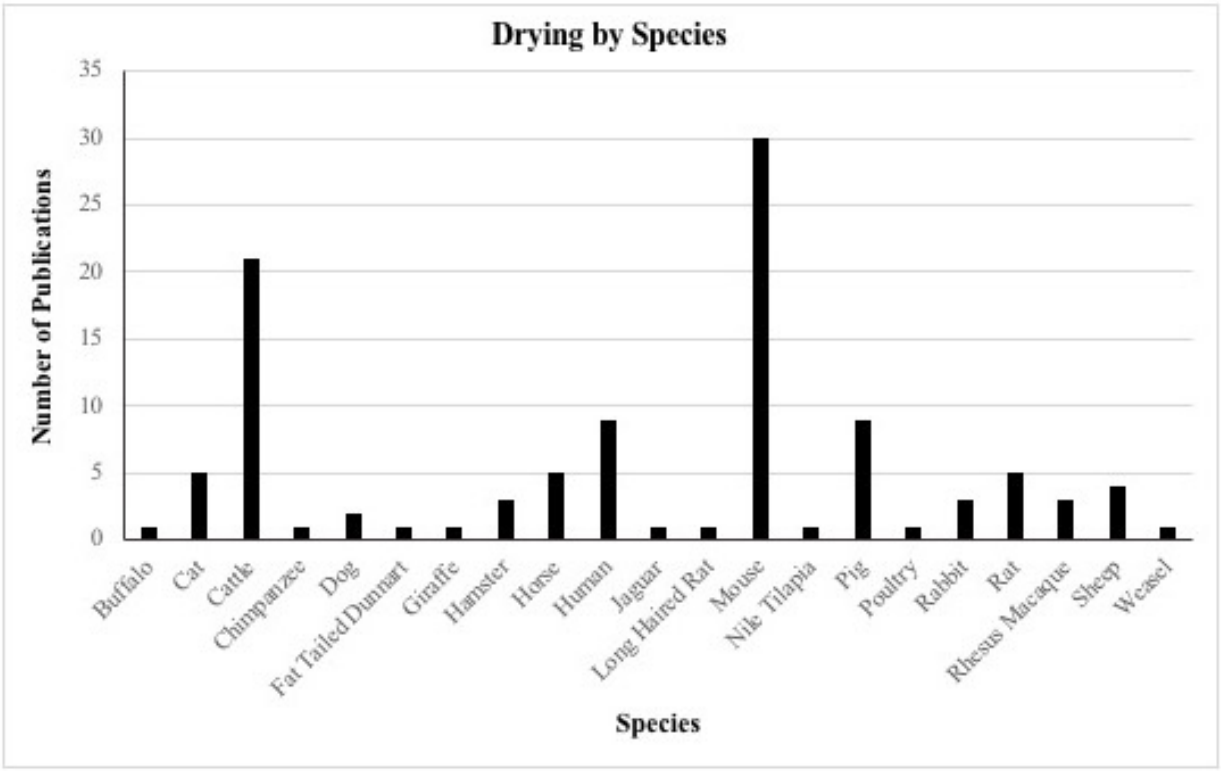
Species	Drying technique	Study endpoint	Reference
Hamster	Freeze-drying	Live offspring	[83]
Hamster	Freeze-drying	MPN formation	[154]
Horse	Freeze-drying	Sperm motility	[155]
Horse	Air-drying	8-16-cells embryos	[156]
Horse	Freeze-drying	Live offspring	[84]
Horse	Freeze-drying	DNA integrity	[112,157]
Human	Freeze-drying	Sperm survival	[70,71]
Human	Freeze-drying	MPN formation	[78,79,154]
Human	Freeze-drying	Chromosome integrity	[158]
Human	Freeze-drying	DNA integrity	[159-161]
Human	Freeze-drying	Membrane integrity	[162]
Jaguar	Freeze-drying	MPN development in mouse oocyte	[150]
Long-haired rat	Freeze-drying	MPN development in mouse oocyte	[150]
Mouse	Freeze-drying	Live offspring	[80,98,163-171]
Mouse	Freeze-drying	Day ≥14 foetuses	[158,172-175]
Mouse	Freeze-drying	Blastocyst formation	[176-178]

Species	Drying technique	Study endpoint	Reference
Mouse	Freeze-drying	DNA/Chromosome integrity	[145,179-183]
Mouse	Partial convective-drying	Live offspring	[91,184]
Mouse	Evaporative-drying	Live offspring	[185-188]
Mouse	Convective-drying	Live offspring	[189,190]
Mouse	Convective-drying	Day 15 foetuses	[175]
Nile tilapia	Freeze-drying	ICSI could not be performed	[191]
Poultry	Freeze-drying	Sperm motility	[66]
Rabbit	Freeze-drying	Live offspring	[73,82]
Rabbit	Freeze-drying	DNA integrity	[99]
Rabbit	Freeze-drying	6-8-cells embryo development	[78]
Rat	Heat-drying	Live offspring	[192]
Rat	Freeze-drying	Live offspring	[81,169,193-195]
Rhesus	Vacuum-drying	Blastocyst formation	[95,196]

Species	Drying technique	Study endpoint	Reference
macaque			
Rhesus macaque	Freeze-drying	Sperm asters, MPN formation	[197]
Sheep	Freeze-drying	MPN formation	[198]
Sheep	Partial freeze-drying	Sperm motility	[199]
Sheep	Freeze-drying	Blastocyst formation	[90,200]
Weasel	Freeze-drying	MPN development in mouse oocyte	[150]

MPN: male pro-nucleus

1044 **Figure 1**



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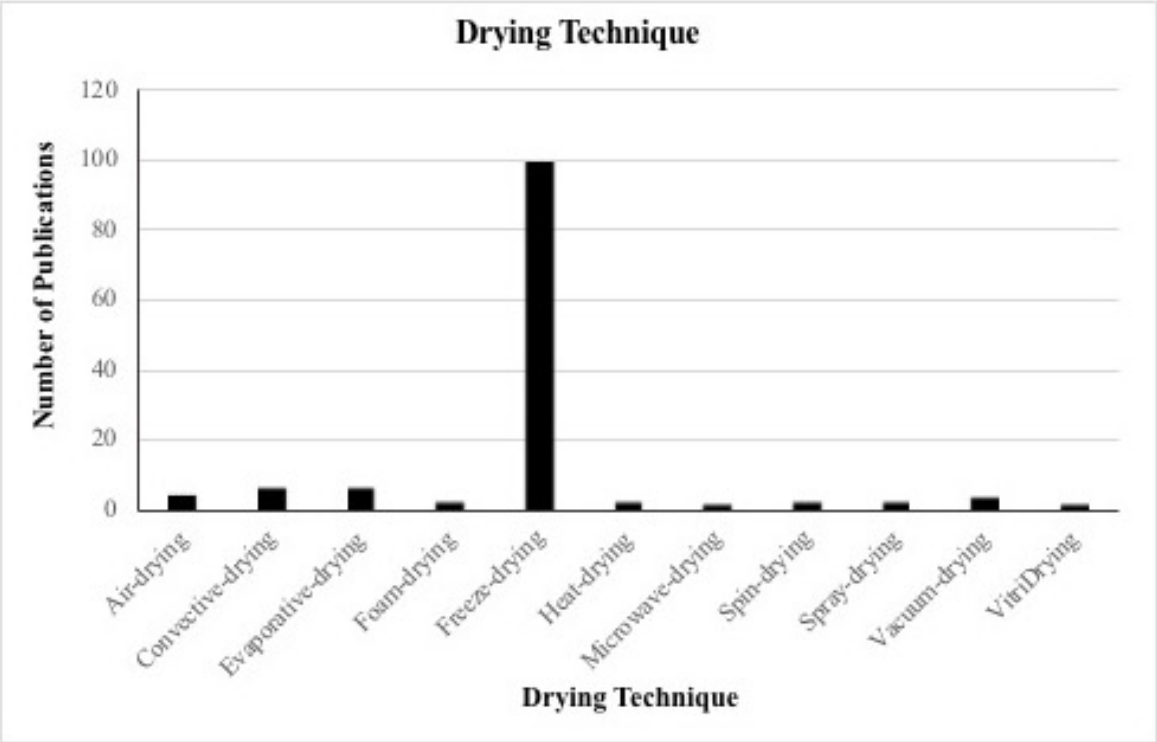
1046 **Figure 2**



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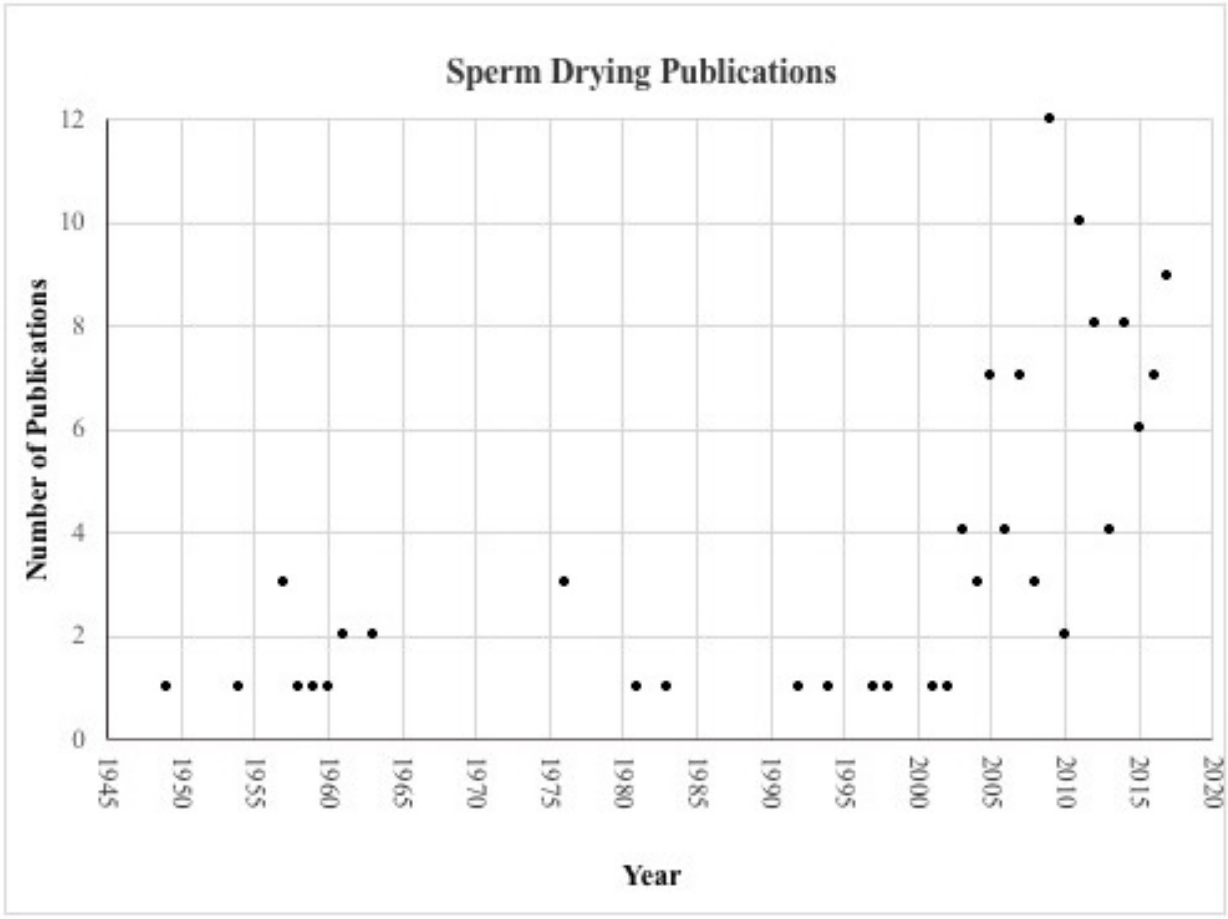
1048 **Figure 3**



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Figures legends

1. **Figure 1**: Number of publications related to cells or sperm drying by species
2. **Figure 2**: Number of publications related to cells or sperm drying by the drying technique.
3. **Figure 3**: Number of publications related to sperm drying over the past ~70 years