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FUNDING STRATEGIES FOR BIOLOGICAL RESOURCE CONSERVATION – EXPERIENCE FROM ENDANGERED SPECIES

Kurt Benirschke

Abstract: *It has been virtually impossible to obtain public funds from the usual granting agencies to support a tissue bank for cells, DNA, or tissues of endangered species. This is true in spite of the National Academy of Sciences publication in 1978: "Conservation of Germplasm Resources - An Imperative." The San Diego Zoo developed a Research Department in 1975 which is currently very active. At this institution, a "Frozen Zoo" was created for cell lines of all sorts of mammalian species, mostly endangered. This bank now contains over 1300 samples and is widely used for research and made available to qualified outsiders. It has been funded by the Zoological Society of San Diego through the foresight and wisdom of its Board of Trustees, who have made a firm commitment to its maintenance. Some monies are recovered from research grants. The National Institutes of Health initially participated in an institutional development grant. This bought some equipment. Nevertheless, the bulk of support has come from philanthropic donations, the "Kicks for Critters" campaign of my son, and the Zoological Society. It is essential that the public and granting agencies be educated to support such essential facilities.*

Kurt Benirschke *was born in Glückstadt, Germany. He was educated in various German universities and received his M.D. from Hamburg University in 1948. He immigrated to the United States in 1949. From 1951 to 1954, he continued his postgraduate education at various hospitals of Harvard Medical School. He was a Pathologist with the Boston Lying-in Hospital from 1955 until 1960. In 1960, he joined, as Chair, the Pathology Department of Dartmouth Medical School, Hanover, New Hampshire, where he remained until 1970. In that year, he joined the faculty of the University of California, San Diego Medical Center as Professor of Pathology and Reproductive Medicine where he continues today. From 1975 to 1986, he also served as Director of Research for the Zoological Society of San Diego.*

This topic should be of concern to all of us as most of the large animals are going to die out very soon. My interest in conserving some of the genomes of large animals arose about 30 years ago when I lived in New Hampshire. I was interested in beginning karyotypic delineation of larger and usually very inaccessible animal species. One day I happened to collect a deer that had only six chromosomes while its closest relative had 46. We were so perplexed that we thought something had gotten into our culture and mutated it so as to fuse all the chromosomes to make six out of 46. It turned out actually that *Muntiacus muntjac*, the Indian muntjac, has only six large chromosomes, while the muntjac from Taiwan, *Muntiacus reevesi*, has 46. In order to do some comparative studies and find out more about similar things, I decided what we really needed to do is store some of these cultures. In the future, many of the comparative studies that we would like to do, particularly if new methodologies come along, will not be possible because we will not have a giraffe or muntjac or any of the other species. It turns out that for most large species, a blue whale, for example, there are no genomes you can access if you wish to have a culture or the chromosomes or anything else. This, unfortunately, is true of virtually all large mammals. With this in mind, I began freezing away fibroblast strains in a very systematic fashion. That is what I want to tell you about very briefly.

I did this initially in New Hampshire at Dartmouth, and when I moved to San Diego I took the cultures with me. They all died in a laboratory accident. The risk of accident is something that has not been talked about, but needs to be addressed in the future for collections such as these. If you have once gone through the trouble of collecting blue whales, gorillas, or orangutans with which you do research, you certainly do not want to have them die because the freezer sprang a leak and let the liquid nitrogen out over Easter.

I also want to address very briefly the problem of getting money for collections. This has been very difficult indeed; it is really what I was asked to speak about. In 1972 or 1973, I convinced the San Diego Zoo that they needed to have an independent research arm to do in-house research. They started in 1975 and I served there for 11 years doing this. During this time, I extracted from the Zoological Society a commitment to look after what has become an increasingly large and more valuable collection of strains of cells as well as spermatozoa and fertilized ova from animals. We have been successful at least locally in the San Diego area in making popular the notion of a frozen zoo. It is a very gross oversimplification, but it has convinced the public that we really need the money to do this. My son, as you may know, was a prominent San Diego Charger, and through the Kicks for Critters Program he was able to raise the sums necessary to maintain and build up the collection. The initial equipment money came from an equipment grant from the NIH obtained when I had a large NIH grant.

The zoo research activities have now been dubbed the Center for

Reproduction of Endangered Species (CRES), and I want to address what this collection consists of, who uses it, what sort of samples are being collected and shipped out, and what the cost of it is (Table 1). The frozen zoo basically is a repository of cell strains, fibroblasts largely, sometimes other cell types, that have been characterized for the most part. That is, almost all of them have had at least karyotypic analysis. They have been subcultured and a variety of vials has been prepared from them. They are relatively early cultures so that there are many further replications possible. They are kept in small vials in liquid nitrogen in duplicate tanks. I am desperately seeking a place where a second replicate collection could be housed away from possible earthquakes or fires. If a proposed animal genetic center at Davis becomes a reality, then we will be able to place a duplicate tank there. Its maintenance would require very little more than periodic checking and liquid nitrogen filling.

Table 1. ***Status of the "Frozen Zoo" of the San Diego Zoological Society***

- 1,350 mammalian cell strains
- 250 species of 11 orders
- 58 genera and 130 species of Artiodactyla
- Permanent records include history of animal and ISIS number
- Record system is computerized
- Genealogy is usually known
- Each culture is usually karyotyped
- Storage is in liquid nitrogen vapor phase, -197°C
- A duplicate collection is kept in a separate tank
- Tanks have alarm systems
- Estimated cost: $19/year/sample

What we have been collecting is virtually everything that has come by our door in the animal world. We have made use of autopsies, and sometimes very fresh animals have been available. In our autopsy room at the San Diego Zoo, all dead animals are autopsied, and from almost all of them we have collected specimens. Those that have grown well and were not somehow contaminated have been placed into the collection. It is a large collection of duplicate cultures very well characterized by species and very often by the point of origin. The genealogy of each culture is indexed in a computer program that lives in the VAX at the University of California and usually with a number in the International Species Identification Program (ISIS) at the zoo in Minneapolis, Minnesota. ISIS has a stock list of all the animals in zoos in this country and in the world, more or less. A reference point from our collection to that collection is available. This is of importance particularly when breeding animals.

Because of our interests and because of the profusion of Artiodactyla at the San Diego Zoo, our collection consists of a large number of Artiodactyla. There are lots of primates and other animals. These are invaluable for the researcher who is working in genetics or evolu-

tion of mammal species. Without our collection, research material would be totally inaccessible. One could not possibly get 10 gorilla or 20 dolphin specimens from anywhere else.

Permanent records are kept in duplicate, in books as well as backed up in a computer. We have our own in the laboratory, and it is connected to the VAX at the University. Most of these specimens are karyotyped and reasonably well defined. We have two of these large tanks, and they are automatically fed with liquid nitrogen. There is an alarm system, but unfortunately both tanks are in the same building. There is no further backup available for any of this. The collections are deep frozen in tissue culture fluid, very well indicated, and located easily. More recently we have some sperm and ova. The freezing takes place under preset conditions by means of a programmable, computerized freezing apparatus. This permits the protocol to be optimized for each species that is frozen.

In our collection are an over-abundance of Artiodactyla, primates, Perissodactyla, or their relatives, a few bats and insectivores, and other little critters which, unfortunately, are not being collected by very many people. There is, of course, the one collection of bats and rodents at Texas A&M University, but, by and large, a greater amassment of these animals is not available in any laboratory to the best of my knowledge. At the American Type Culture Collection (ATCC) the mammalian material is mostly human cell lines. We have tried to collaborate with ATCC, and I hope this will work in the future more satisfactorily. They are not really interested in storing a large number of lines from different mammals, and there is no one else at the moment that does so. T.C. Hsu at Texas once collected some lines, but most of these he has sent away, having gone into cancer research.

The current supervision of the CRES activities is by Oliver Ryder who has a Ph.D. in molecular genetics with a great interest in comparative genetics and evolution. The collection continues to be supported by the Zoological Society of San Diego, largely through its Center for Reproduction and Endangered Species. Much of the actual work going on with the collection is being funded by research grants that contribute a few dollars here and there, but that is not the major portion of it. In calculating the real costs, it is very difficult to arrive at a figure for storing a sample per species per year. We have come up with about $19 a year. That may seem very high since the sample only sits in liquid nitrogen. But this is the actual cost of freezing it down, unfreezing it, checking it out, karyotyping it, refreezing it, verifying it every now and then, and entering it into the computer.

We are continually being asked by many investigators to ship them tissues of animals that are virtually unattainable by them. This is a major problem for us, because it takes time away from our own research activities. We are not there to make money on it; in fact, our billing for shipping is very improper and not very well handled. The real problem is international shipment of animal tissues and the im-

porting of tissues from other countries. I have personally a great interest in South American animals, particularly Artiodactyla, and find it virtually impossible to bring material from South America due to USDA restrictions. Likewise there are great difficulties bringing anything in from Africa and other countries. One should abide by the legal constraints put upon us. None of us would like to bring in hoof-and-mouth disease viruses or other agents that are potentially dangerous if disseminated throughout the world. It is for this reason, in part, that I think our collection is very important. It was established from animals that were already here and were post quarantine and certified not to have hog cholera or some other dreadful disease that might raise havoc with livestock in this country.

We use the collection extensively for the interpretation of the cytogenetic evolution of animals. More importantly, we have used it for studying the ever-increasing problems of infertility that we see in zoological parks. One of my more interesting recent observations has been when we tried to characterize Soemmerring's gazelle (*Gazella soemmerringi*) which comes from Somalia. In collections they have died out regularly after a few crosses. I collected specimens of Soemmerring's gazelles and karyotyped them. There turned out to be nine different chromosomal variants in these specimens. That is, there were numerical chromosomal variants, in addition to major translocations which conferred sterility on the offspring. In fact, we have no idea what the karyotype of Soemmerring's gazelle actually should be, because we have nine different karyotypes from animals identified by curators as Soemmerring's gazelles which allegedly come from the Somalian highlands.

At the moment we are studying dik-diks, some of which are monotypic, monospecific actually. Dik-diks have at least four different chromosomal variants which render them subfertile or infertile. For the breeding of animals in the future it is essential that one have this information, otherwise one will never get self-sustaining populations of animals that have become extinct in the wild or are no longer obtainable.

We have a number of guest investigators and ship material out to other researchers. Moreover, recently the American Zoologic Society and the American Association for Zoological Parks and Aquariums have recognized the necessity to form species survival plans for the animals that are almost gone. These include several 'emblem' species, but there are many others needing attention. For them we are doing a lot of different types of work. At the moment our work is only in mammals, but we hope in the future it will be in birds as well. We are beginning, for instance, with the California condor, to fingerprint its genome.

One thing has been troubling me a lot. I have the ability only to freeze mammalian cell lines. I am not a cryobiologist; I know nothing about the ability or lack of ability to freeze avian cells. There are many

large birds like the California condor whose genome we would like to study in the future, but I know nothing about our ability to freeze them. The same goes for reptiles. Such expertise needs to be developed in the future. I would hope it could occur in a collection center like that proposed at Davis.

In any event there are many future uses for the collection. They are challenging in the current context of better understanding the DNA of mammals and their evolution. It is important to have available at least the DNA in the form of replicating cells. It is better than having nothing in the future. We would like to make DNA libraries and have done a few, but we have not even begun to scratch the surface on a systematic basis.

It is true that the public, by and large, looks upon the content of a frozen zoo as something from which you can make rhinoceroses or gorillas after the rhinoceroses or gorillas have become extinct. They look upon this as a wondrous machine with which to reproduce animals in the future. Unfortunately, some of the publicists in our zoo and in general have misled the public in this respect. It is true we are trying to learn how to freeze, say, rhinoceros semen or semen from giraffes. But in my own view this is not a very likely success story for the future, because there will be no more rhinoceroses or giraffes into which one can inject the semen. It would be very difficult at the moment to collect semen of Sumatran rhinoceroses because there are only four in captivity (one male only), and nothing is known about their biology. Most curators in most zoos would not allow one to touch one of those animals for electro-ejaculation or other purposes. So for the future of the systematic collection of genomes that are potentially replicating, such as semen or fertilized eggs, the Center is not yet ready. I think the world is not ready. At the moment we are trying to conserve primarily DNA and cell strains so our colleagues in the future have it available.

DISCUSSION

Question: ***Which phase of nitrogen is used for storage, the liquid or vapor phase?***

Benirschke: Actually the storage occurs above the liquid in the vapor phase. But it is automatically fed to a level that we predetermine. The survivability of these strains is very good indeed.