Biotechnology

introduction

The guidelines of the **Biotechnology Program** are research and development aiming at developing and manufacturing products of pharmaceutical interest. This Program has two main research areas, namely Pituitary Hormones and Biopharmaceuticals.

The first one comprises a group with a long experience on Recombinant Human Pituitary Hormone synthesis, purification and characterization. Up to now they have worked mostly with human growth hormone (hGH), human prolactin (hPRL), human thyrotropin (hTSH), human follicle stimulating hormone (hFSH) and human luteotropin (hLH), with a particular emphasis on glycoprotein carbohydrate structures. An important research line is devoted to Growth Hormone Gene Therapy, working mostly on animal models: immunocompetent and immunodeficient-dwarf mice. For several years this development has been based on *ex vivo* grafting of transduced keratinocytes, while more recently very promising results have been obtained with the injections and electroporation of naked plasmid DNA. Besides research, they have also activities in the Biotechnological Production and Downstream Processing of the same recombinant hormones, which are produced in both *E. coli* and mammalian cells and in the development of joint-ventures with the National Industry. The biological effects of radiation on cells are also studied, specially concerning the administration of ^{131}I together with thyroid-stimulating hormone in thyroid cancer.

The Biopharmaceutical area is dedicated to the research of isolation, structural analysis and biological activities in different biological systems of macromolecules. These macromolecules are peptides or proteins, either native or recombinant with medical or pharmaceutical interest. During this period new proteins related to serine protease activity, breast cancer development and angiogenesis were described. The effects of ionizing radiation on macromolecules have also been investigated to detoxify animal venoms in order to improve antigens for anti-sera production, or even modify microorganisms for vaccination. Recently, we started investigating the peptide fractions of several venoms, identifying many serine-protease and metalloprotease inhibitors. Ideally, these inhibitors will be co-crystalized with the target enzyme, aiming to characterize the inhibitor-enzyme interaction. Such data could provide knowledge to develop new drugs against coagulopathies and other endogenous protease related diseases.

The Animal Laboratory Division of IPEN is responsible for the breeding and production of small laboratory animals. In this facility Specific Pathogen Free (SPF) animals are bred and maintained, under controlled sanitary conditions, to be used for testing of the radioisotopes production and research. This facility also produces different mutant mice, severely immunodeficient mice and their offspring, besides other mice lineages as well as normal rats.

The research and production activities related to the five human pituitary hormones, namely growth hormone (hGH), prolactin (hPRL), thyrotropin (hTSH), follitropin (hFSH) and luteotropin (hLH), still constitute the basic working field of the group. Our goal is the development of applied research, especially emphasizing the interaction between the Academic and the Industrial world, an aspect that has been neglected for so long in Brazil. Under this aspect the joint-venture already set up at the end of 2007 with a successful biotechnology company (FK-Biotecnologia), that demonstrated great interest in hormone and antibody production for diagnostic and therapeutic applications, has continued. At the same time new collaborations have been set up with the Aarhus University (Denmark) and with clinicians from FMUSP, especially in the field of Gene Therapy, and with the group of Genetic Ichthiology of the ICB-USP for the purpose of cloning *Arapaima gigas* (Pirarucu) gonadotropins. As always, the main emphasis of the group has been given to scientific production and to collaboration with well- known national and international research groups. Thus, in this 3-year period, 19 scientific papers have been published all in international journal whose impact factor was always between 1 and 5.3, and 5 abstracts published in journals of high impact.

Human growth hormone (hGH)

Human growth hormone (hGH) production and quality control has been already established at the laboratory level and, as stated several times, is only waiting for the "industrial decision", that unfortunately is frequently independent from the researches. However, an important research line has being carried out developing alternative *ex vivo* and *in vivo* Gene Therapy strategies for phenotypic correction of dwarfism, using the human (hGH) and the mouse (mGH) genes. In these studies, two animal models available at our animal facility, the immunocompetent dwarf (lit/lit) and the immunodeficient dwarf (lit/scid) mice, are employed. The goal of this approach is the development of an animal model, based on these dwarf mice, in which it can be possible to obtain useful and sustained circulatory levels of growth hormone with phenotypic corrections, such as body weight gain and longitudinal growth. Our researches are thus moving closer to pre-clinical testing. More recently, the efforts were concentrated on the use of an *in vivo* system based on naked hGH DNA administration followed by electroporation in the quadriceps muscle of lit/scid mice. In a previous work, we related a sustained secretion of hGH during a 60-day assay together with a highly significant increase in the body weight of these animals. In the period of this report, we published another paper where the growth and the paracrine/endocrine effects of this type of administration are compared to those obtained after regular injection of recombinant hGH (Higuti E *et al*., 2012). The main conclusion was that the single hGH-DNA administration was comparable to repeated hormone injections for promoting growth and may represent a feasible alternative for the treatment of GH deficiency. In this period, we also developed a novel homologous model, in which the mGH gene was electrotransfered to the immunocompetent mice (lit/lit), a condition more similar to that of GH-deficient children. The results of this work were recently published (Cecchi CR *et al*., 2014) and they also confirmed the feasibility of the proposed treatment, both in terms of higher circulatory levels of the main effector of GH, the insulin-like growth factor I (IGF-I), and the absence of anti-GH antibody formation. We also can emphasize the continuity of our participation to the most prestigious International Meetings in this area, such as the XIX and XX Congress of the European Society of Gene and Cell Therapy, in England and France, 2011 and 2012, in which we participated with four abstracts published in the Human Gene Therapy and the XXI Congress of the European Society of Gene and Cell Therapy, in Spain, 2013, with one abstract in the same journal. These studies are being carried out in collaboration with the Endocrinology Division of the FMUSP/São Paulo and with the Department of Biomedicine at Aarhus University, including in this period the beginning of our participation to the Gene Therapy Initiative at Aarhus, supported by a Danish Foundation.

Considering that hGH is positively influencing muscular dystrophy, a collaboration with Dr. Mayana Zatz from the "Centro de Estudos do Genoma Humano" IBUSP was set up. Dystrophic mice of four distinct mutated strains obtained from the Jackson Laboratory (Maine, USA) were maintained in the animal house of the Biotechnology Center. A series of mating were carried out and techniques for the determination of dystrophy and growth hormone deficiency by DNA sequencing were established. Other activities were also carried out under this collaboration, based on the *in vivo* use of human mesenchymal stromal cells combined with IGF-I (Secco M et al., 2013).

Human pituitary glycoprotein hormones

Human pituitary glycoprotein hormones include thyrotropin (hTSH), follitropin (hFSH) and luteotropin (hLH), all heterodimers formed by an alpha and a beta subunit. This hormone is related to thyroid function and metabolism, and is used in the diagnosis and therapy of thyroid cancer, while hFSH and hLH are mostly used for the treatment of human infertility. These recombinant products are among those with the highest aggregate value, their purified forms reaching prices up to US$ 12.000/mg! Considering their carbohydrate moiety, which is strictly related to their in vivo bioactivity, these proteins must be synthesized in mammalian cells, the most commonly used for their industrial production being CHO cells. Our laboratory has synthesized and characterized hTSH, having also the know-how for synthesizing hFSH and hLH. During this period (2011-2013), the laboratory completed the studies concerning to the substitution of animal-based bioassays by alternative methodologies for hFSH and hTSH potency assessment. A suitable physical-chemical testing strategy (RP-HPLC) was proposed, thus avoiding or reducing animal use (Fig. 1) (Almeida BE *et al*., 2011). An important issue in biopharmaceutical drug quality control is the availability of chromatographic tools that permit the accurate analysis of the active principle without interference from other components of the final drug formulation with similar hydrophobic, ionic and/or spectrophotometric characteristics. Aiming at an efficient and careful quality control, three RP-HPLC methodologies were developed for the qualitative and quantitative analysis of hTSH, hFSH, hCG and hLH in the presence of substantial excesses of human serum albumin (Almeida BE *et al*., 2012). A systematic investigation of aspects involved in the production strategy of recombinant hTSH, a bioactive glycoprotein, was also carried out. The effects of butyrate and manganese on productivity, sialylation, N-glycosylation site occupancy and biological properties of CHO-derived hTSH were evaluated, showing no evidence of alterations in its bioavailability, although increases in hTSH production (up to 3-fold), in sialylation (up to 14%) and site occupancy (up to 3%) occurred (Damiani R *et al*., 2013).

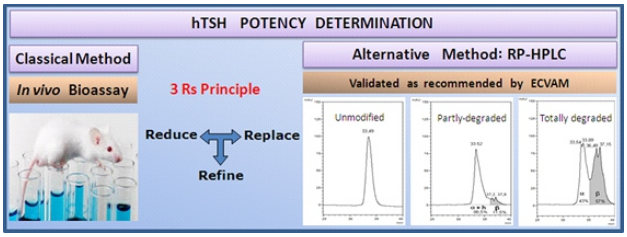

Figure 1. Classical and alternative method for hTSH potency determination.

Pirarucu (*Araipama gigas*) pituitary gonadotropins

Araipama gigas (pirarucu) is a giant fish native to the Amazon River basin that can reach 2 meters in length and weigh up to 150 Kg. This species is in danger of disappearing because of the exploitation by the fishing industry and increasing human presence. It is largely used for food and extractivism and commercial breeding is still incipient. Our research group has isolated, from the pituitaries, and characterized the cDNA of the α-subunit of *A. gigas* (ag) gonadotropins (FSH and LH), carrying out also a phylogenetic analysis based on its amino acid sequence (Faria MT *et al*., 2013). We are now working for obtaining the cDNAs of ag-FSHβ and ag-LHβ-subunits. The biotechnological synthesis of these hormones, useful for physiology and fertility applications, will be carried out with basis on these isolated genes.

Prolactin (PRL)

Prolactin is a 23 kDa protein hormone secreted by the anterior pituitary. Its therapeutic use is still limited, while it is important for diagnostic applications such as prolactinomas, infertility and

tumorigenesis. In the context of recombinant hormone production, the group has been developing the expression, purification and characterization of human prolactin and its analogs and antagonists, namely S179D-hPRL and G129R-hPRL, in a research laboratory scale. In order to scale up the production in Chinese Hamster Ovary cells, a fast and practical method for adaptation of this cell expressing hPRL to serum-free suspension culture was conducted in spinners. The adaptation from adherent cells to suspension took only 40 days. The daily collected medium was purified and the glycosylated and non-glycosylated forms of hPRL were obtained with the expected biological activity (Arthuso FS *et al*., 2012). Glycosylated human prolactin (G-hPRL), obtained from CHO cells adapted to suspension and treated with cycloheximide, was also compared to the native pituitary form of G-hPRL for what concerns carbohydrate structures. A novel method of N-glycoprofiling analysis was set up and a manuscript was submitted to Glycobiology. Another study aimed at producing mouse prolactin in *E. coli* bacteria. It is important to conduct future studies with prolactin in a homologous system as most of experiments are done first in mice. This hormone in its authentic form, without initial methionine, is expressed in the periplasm using the lambda PL promoter in a constitutive way. Although the expression levels are extremely low (2.2 µg/mL) in a bioreactor using 5 liter medium, with poor mass fraction, the purification via a three-step laboratory process was carried out efficiently and a bioactivity of 39.5 IU/mg in Nb2 cells was determined against the International Standard of recombinant hPRL (WHO) (Suzuki MF *et al*., 2012).

Cellular response to ionizing radiation

Ionizing radiation is a physical agent known to induce mutation and cancer, being also used as a widespread therapeutic modality for cancer treatment. Thus, one of the challenges in radiobiology and oncology is to understand how the cells respond to oxidative stress resulting from exposure to radiation and the pathway they will follow. On the basis of the above considerations, the group of cytogenetic/mutagenesis has developed studies focusing three interlinked aspects. The objective of the first one (cytogenetic aspect) consists of a comparative study of the effects of different radiation types and radionuclides of medical interest (^{153}Sm, ^{177}Lu, ^{131}I, ^{18}F, ^{68}Ga), in human and rodent cells, by cytogenetic and biochemical techniques. The second aspect of our study consists in the establishment of dose-response curves for different types of radiation (γ, β and neutron) for biological dosimetry (dosimetric aspect) directed to the quantitative estimate of absorbed dose, according to the criteria adopted by IAEA (2001). The calibration curves for the γ radiation of ^{60}Co and ^{137}Cs, for the β radiation of ^{90}Sr and for fission neutrons produced in the Reactor R1 of IPEN-CNEN/SP have already been established by assessment of chromosome aberrations, micronucleus and comet assays (Fig. 2).

An improved *in vitro* micronucleus assay for biological dosimetry was introduced in our laboratory utilizing fluorescent staining technique on human tumor cells (MCF-7) treated with a nitric oxide inhibitor and irradiated (^{60}Co), This showed that nitric oxide inhibition could be a radiossensitizing approach in tumor therapy, increasing radioinduced genotoxic damage and reducing cell viability and clonogenic potential. A third aspect (therapeutic approach) consists in evaluating the cytogenetic effects of radiopharmaceuticals used in nuclear medicine, e.g. ^{131}I, administered to patients with differentiated thyroid carcinoma (DTC), with or without use of recombinant human thyrotropin (Thyrogen® or rhTSH) (project approved by FAPESP), that is being carried out in collaboration with the Nuclear Medicine Center of FMUSP. A study also using human thyroid cancer cells (WRO) is ongoing for comparing the radiosensitivity between target cells of radioiodine (thyroid cells) and peripheral lymphocytes of patients with DTC. Included in this research area is also a collaboration study joining researchers from the Centers of Biotechnology and Radiopharmacy and the Biosyntesis Laboratory for pre-clinical trials of radiopharmaceuticals produced at the Radiopharmacy Center (IPEN-CNEN/SP). This work is currently being supported by an institutional grant, and final results will be collected until May, 2015. Finally, our research also includes the study of radiomodifiers from natural sources, such as resveratrol (in collaboration with Chemical and Environmental Center) and propolis. The study about the radiomodificator effect of propolis and their HPLC purified fractions (in collaboration with a Japanese group) in normal and human cancer cells is in progress, having already published data on the effect of propolis on CHO-K1 cells irradiated with ^{60}Co through the differential staining technique and digital analysis. Using this technique we could be able to analyse 10-100x more cells than by the usual manual counting. The data obtained via genotoxic and cytotoxic tests and survival curves showed a radioprotector effect of propolis on the induction of DNA damage and cell death was submitted to Mutation Research. These data indicate a potential promising use of propolis as a natural, non-toxic, effective substance for protection against genotoxic and cytotoxic damages induced by the ionizing radiation.

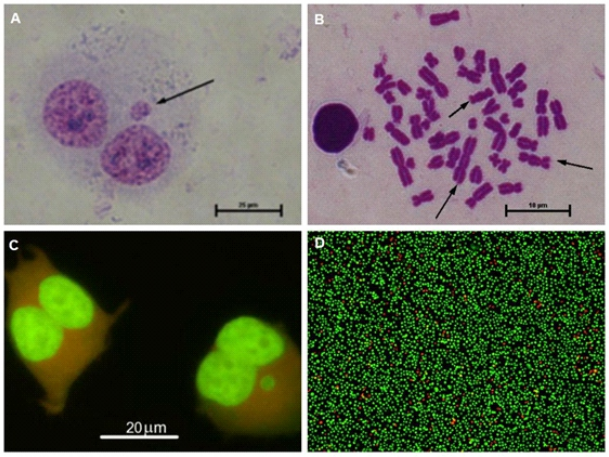

Figure 2. A) Binucleated cell with one micronucleus (Giemsa staining); B) Chromosome aberration (Giemsa staining); C) Binucleated cells with micronucleus (fluorescent staining) and D) Viable (green) and necrotic (red) cells (differential fluorescent staining). 10x.

Protein radioiodination

The Group of Hormones of the Biotechnology Center has a long and well-known expertise on Protein Radioiodination, mostly with the use of ^{125}I. At least ten studies on pituitary hormone radioiodination have been published, before the year 2000, in International Journals of high impact. In the period 2001-2010, nine more papers have been published, providing precious collaborations to other Brazilian research groups, in prestigious journals as well. During the period 2011-2013, object of the present Progress Report, one more paper has been published in Journal of Experimental and Integrative Medicine, in collaboration with the Department of Pharmacology of Unicamp (Campinas - SP). This excellent specialty, in a specific area of the Nuclear Field so long neglected, deserves a proper emphasis, especially considering the work of all the technicians and researchers that dedicated themselves, to the study and manipulation of this extremely useful radioisotope.

Identification, isolation and characterization of new compounds of clinical interest, from plants and animal molecules have been developed. Additionally, studies involving ionizing radiation have been done in order to detoxify or even modify molecules mainly to improve antiserum production and vaccines. Many of pre-clinical assays, the main tool, employed to characterize the candidate to a new medicine have now been standardized to get an accreditation.

Biological screening of snake venoms and toxins

Snake venoms are an extremely rich source of biologically active substances modulating several aspects of the regulation of homeostasis. The investigation of potential new drugs in biological samples has been a major field of research in many laboratories. This activity has focused part of its efforts in screening and isolating toxins with potential therapeutic uses. Amongst those, toxins from the Brazilian rattlesnake which seem to be involved in the down regulation of the sodium/potassium pump have been investigated. Such molecules have a potential for further understanding the fine physiology of many cell types and for guiding the rationale design of cellular metabolism modulators. During investigation of the action mechanism of snake neurotoxins, we developed a model of excitable medium, the Belousov-Zabotinsky reaction, was developed. It allows understanding many aspects of neuronal transmission and membrane physiology. Among several experiments a set of reactions to microgravity, using a sounding rocket provided by a collaboration between Brazilian and German Space Agencies was done, and it was possible to observe that, indeed gravity seems to be able to act at molecular levels, modulating reactions which can be compared to those ruling neuronal transmission, onset of migraine and epileptic crises and regulation of heart beating. Also toxins with modulatory activity on the blood clotting system (Snake Venom Serine Protease - SVSP) are under investigation. Such toxins might be employed for the treatment of coagulation disorders and as auxiliary drugs in major surgeries where unexpected activation of the blood clotting cascade could put the patient at risk. These proteases are also key players in a wide range of biological processes; for example, in regulating the cell cycle, cell growth and differentiation, affect the haemostatic system, antigen processing and angiogenesis.

In addition, it is becoming apparent that the aberrant functioning of certain proteases may be involved in several disease states, including Alzheimer's disease, in cancer metastasis and in inflammation (over-reactive inflammatory reactions in CNS often cause irreversible neuronal damage). In this period it was reported the molecular cloning of five new nucleotide sequences of SVSP (GenBank accession number AY954040 EU360951; EU360952; EU360953; EU360954) that were retrieved from a cDNA library constructed with the venom gland of a single specimen of Brazilian rattlesnake *Crotalus durissus terrificus*. These sequences have been analyzed in silico with respect to their cDNA organization, similarity in relation to others SVSPs, their probable biological functions and the overall particularities of these nucleotide sequences. The functional dendrogram was generated to group the serine protease activities in relation to others snake venom thrombin-like enzymes. Moreover, a rapid and efficient method for screening vectors for mammalian cell expression was developed. It is based on the fact that SVSPs are difficult-to-express toxins since they contain several disulfide bounds and are glycosylated. The biochemical properties and the molecular weight of recombinant toxin were compared to native gyroxin purified from the venom and are essentially identical.

Biological evaluation of new products for health

This activity is mainly based on the biological evaluation of substances and biomaterials performing *in vitro* and *in vivo* tests. These tests are carried out in compliance with the rules of ISO-10.993 and some other international directives. Such tests include: cytotoxicity, genotoxicity, hemocompatibility etc and some others tests of systemic toxicity and implants. Synthesis of polymeric biomaterials was also done, resulting in three patents submitted to INPI in 2007. A biomaterial can be defined as a substance (with the exception of drugs) or a combination of substances (either synthetic or natural), employed in the treatment, improvement or substitution of organism tissues, organs or function. Since interaction with the biological system is involved, biocompatibility implies the capability of the material to exhibit in the host the appropriate functional and "biomimetic" qualifications. In recent years, interest in biomedical applications of natural and synthetic polymers has grown steadily, with a substantial contribution to the quality and duration of human life. Presently, novel porous biologically active composites based on hydroxyapatite (HA) and poly(caprolactone) (PCL) have been developed and tested, with potential for use in scaffolds for bone tissue engineering. The experiments are focused on the synthesis and biological response of bone to the PCL/HA composite. Such work resulted in a partnership with the Biosintesis Company which received a financial support from FAPESP (PIPE project).

Recombinant proteins - Refolding from inclusion bodies using high hydrostatic pressure

Until the 1980s decade, the production of proteins for therapeutic and research purposes was obtained by purification from their native sources. The production of proteins was greatly facilitated by transgenic protein expression, overcoming the difficulties of purification of proteins at low levels, presenting low stability, or highly contaminated. The bacteria *Escherichia coli* is the most efficient and cost-effective host for recombinant heterologous protein production. However, *E. coli* is often unable to fully process the recombinant foreign proteins during overexpression and therefore misfolded proteins forms insoluble aggregated proteins in bacterial cytoplasm, known as inclusion bodies (IB). Refolding is necessary to produce active proteins from IB. Utilization of high hydrostatic pressure is a novel and robust method to disaggregate proteins from IB, by solubilization of the aggregates in mild conditions, maintaining the existing native-like secondary and tertiary structure of the insoluble and mostly inactive proteins produced in bacteria. We demonstrated that high pressure can convert insoluble aggregated proteins from IB to preparations with native tertiary structure and fully biological activity with very high yields. Among the proteins that have been successfully refolded by our group are the antiangiogenic and anticancer mammalian protein endostatin, green fluorescent protein, a promising protein for *Shistosoma mansoni* vaccination (Sm29), subunit B of cholera toxin (CTB), among many others. Aiming to improve the quality of proteins and enhance the refolding yields, we are studying the effect of high pressure on proteins, through the determination of intrinsic and extrinsic fluorescence. It is in progress a collaboration to determine the effect of high pressure on endostatin, as verified by Nuclear Magnetic Resonance technique.

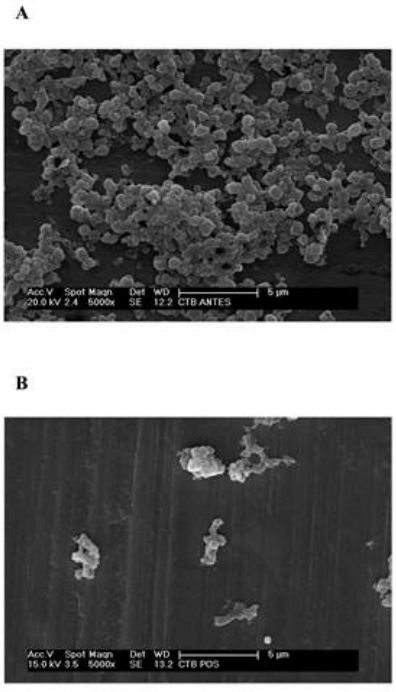

Figure 3. Cholera toxin expressed in *Escherichia coli* inclusion bodies before (A) and after (B) refolding with high pressure. Scale 5 µm.

Structural analyses of soluble human QM protein
The ribosomal protein QM belongs to the L10 family of ribosomal proteins, which is highly conserved from yeast to humans. The presence of the QM protein is necessary for joining the 60S and 40S subunits in a late step of the initiation of mRNA translation. This protein has been identified as a putative tumor suppressor. We investigated the effect of growth temperature on the expression of the soluble form of the 24.5-kDa QM protein. QM was expressed in a soluble form in culture at temperatures of 25°C and 30°C for 16 h. Structural analysis of the soluble protein fraction by circular dichroism showed that this protein has less alpha helix than beta sheet, and a fluorescence assay with zinc incorporation showed that this fraction displays tertiary structure, which has been described previously in the literature. The circular dichroism and fluorescence analyses were made in National Laboratory of Synchrotron Light, Brazil. QM protein binds to c-Jun oncoprotein inhibits its action. This c-Jun protein was expressed, purified and characterized in our laboratory. Another study we are developing is the cloning and expression of the catalytic sites of angiotensin converting enzyme I. These catalytic sites are inhibited by lisinopril, an antihypertensive.

Biological effects of ionizing radiation
Biological effects of ionizing radiation in aqueous solution, produces several highly reactive species. The most important are hydroxyl radical and hydrated electron. These products interact with peptides and proteins causing several modifications such as fragmentation, aggregation or oxidation, which are responsible for detoxification or even few modifications on proteins. These properties of ionizing radiation make it a good tool to improve antiserum production and vaccination process. Additionally, some substances called scavenger can be used to modulate these effects. It was found that the irradiated protein could be selectively incorporated to the cells, due to specific receptor for oxidized protein, the scavenger receptors. This increased uptake could also result in better antigen presentation and high immune response, either humoral, as demonstrated with purified crotoxin or cellular, as recombinant *M. leprae* Rp 18 heat shock protein. Ionizing radiation can also modify biological and structural properties of toxins as crotamine, used here as a model. Biological alterations occurred in irradiated crotamine were observed with spectrophotometric assays.

The Animal Laboratory Division is a facility having 1040 m^2 of built area, distributed in production and stock areas of animal models for IPEN as well as for other institutions. Some of these models bred in this division are unique in Brazil, thus providing extremely useful tools for many investigators. The goal of this division is to act as an animal breeding and experimentation facility, sterilizing products and providing services to guarantee the genetic and sanitary quality of animals employed in investigations focusing mostly on the development of new drugs and radiopharmaceuticals (Fig. 4).

Figure 4. Specific Pathogen Free animals kept under genetic, sanitary and environmental controlled conditions.

Besides breeding animals for use in our institution, this facility also sells animals for other laboratories and offers housing of special care requiring mice and rats upon request. For further information, contact nnascime@ipen.br

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Honor Mention and Awards

The Brazilian Society of Science in Laboratory Animals (SBCAL) honored the work "Influence of ionizing radiation on wellbeing of animals producing anti ophidic serum", realized by Nanci do Nascimento, Miriam C. Guarnieri, Pedro C.L. Oliveira and Roberto Rogero, during the XI Brazilian Congress of Science in Laboratory Animals and the II Forum of Ethic Committee on Animal Use.