# Biotechnology


*Research aiming to develop and manufacture products of pharmaceutical interest*

# Biotechnology

## Introduction

The guidelines of the Biotechnology Program are research and development aiming to develop and manufacture 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) and more recently with human follicle stimulating hormone (hFSH) and human luteotropin (hLH). 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” grafiting 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, especially 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 metallo-protease inhibitors. Ideally, these inhibitors will be co-crystalyzed 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.

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## Pituitary Hormones

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 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 clinicians from FMUSP, especially in the field of Gene Therapy, and with EMBRAPA, for the purpose of cloning Arapaima gigans (Pirarucu) gonadotropins. As always, the main emphasis of the group was 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 7.5.

### 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 sometimes independent from our will. However, an important research line in Gene Therapy has been carried out using the hGH gene. In this period, our group gave continuity to the research in the Gene Therapy field, using the human (hGH) and the mouse (mGH) growth hormone genes. The goal of this approach is the development of an animal model, based on immunodeficient dwarf (lit/scid) mice, in which it can be possible to obtain useful and sustained circulatory levels of these hormones together with phenotypic corrections, such as body weight gain and longitudinal growth (Journal of Gene Medicine, 2008). 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. An important paper was published (Journal of Gene Medicine, 2010), that describes a sustained secretion of hGH during a 60-day assay together with a highly significant increase in the body weight of these animals. We are preparing another manuscript where the paracrine/endocrine effects of this type of administration are compared to those obtained after regular injection of recombinant hGH. In this regard, the two different strategies provided a similar response in terms of weight variation, when comparing the slopes of both growth curves. We also can emphasize our participation to one of the most prestigious International Meetings, such as the "12th Annual Meeting of the American Society of Gene Therapy" in San Diego, 2009, which originated a publication in Molecular Therapy and to the "Gordon Research Conference on Bioelectrochemistry" in 2010, together with a presentation at the "6th Conference on Recombinant Protein Production" in Vienna. Recently, we also started working with lentiviral vectors, carrying the hGH or mGH gene, which can be used to transduce keratinocytes *ex vivo* and transplant these genetically modified cells into the same animal model. This attempt, which was already studied in our laboratory with retroviral vectors, also intends to improve the sustainability of *in vivo* hormone secretion. These studies are being carried out in collaboration with the Endocrinology Division of the FMUSP/São Paulo. 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 adipose multipotent mesenchymal stromal cells (Stem Cells, 2008; Stem Cell Reviews and Reports, 2010).

### 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 (2008-2010) the laboratory completed the study concerning recombinant hTSH synthesis in a reduced $CO_2$ environment, which led to a higher productivity, characterizing for the first time the N-glycan structures of our recombinant preparations in comparison with the only commercial preparation available: ThyrogenR from Genzyme (Molecular Biotechnology, 2008). Aiming at an efficient and careful quality control of biopharmaceuticals, we studied and characterized the individual subunits (alpha and beta) of the three recombinant and pituitary glycoprotein hormones (hTSH, hFSH and hLH), obtained via a particularly efficient and mild dissociation technique of the intact heterodimeric hormones. The 12 different subunits were characterized comparatively for

purity, hydrophobicity, molecular mass and charge distribution by HPLC, mass spectrometry, SDS-PAGE and isoelectric focusing (Journal of Chromatography A, 2009). For the first time a human-like sialylated recombinant hTSH, with sialic acid bound to galactose by both alpha2,3 and alpha2,6 linkages, was synthesized (Protein Expression and Purification, 2009). The new product which was obtained by genetic modification of the carbohydrate moiety, was found similar to traditional recombinant hTSH for what concerns molecular mass, distribution of charge isomers, hydrophobicity and bioactivity, although several differences were observed in N-glycan structures. The *in vivo* influence of these modifications on the pharmacokinetic properties are now being studied. Finally a new RP-HPLC methodology, related to hLH and hCG identification, characterization and qualitative and quantitative analysis of 11 preparations of different origin (pituitary, urinary and recombinant) was also set up (Journal of Pharmaceutical and Biomedical Analysis, 2010). Our laboratory also participated of an International Collaborative Study organized by the World Health Organization (WHO) for the definition of the new International Standard of Recombinant hFSH for bioassay being, together with our collaborators from the Federal University of Santa Maria (RGS), the only Latin American group and also the unique to determine the mass content of hFSH by physical-chemical methods. It should be emphasized that for the case of recombinant glycoproteins, while the protein moiety is identical to the natural, the carbohydrate moiety is necessarily different. Such difference must be studied and evaluated, especially considering the repeated parentheral use of these biopharmaceuticals. More work is in progress concerning this aspect, especially considering the comparison of the N-glycan structures of the recombinant proteins with those of the native ones. Some of these studies are being carried out in collaboration with the University of Oslo (Norway) and of Vienna (Austria). Also more work is in progress related to the substitution of *in vivo* bioassays with physical-chemical techniques and to the cloning and synthesis of Arapaima Gigans (Pirarucu) gonadotropins, in collaboration with EMBRAPA, aiming at increasing the fertility of this species for alimentary purposes.

### Prolactin (hPRL)

Prolactin is the second (after hGH) unmodified protein hormone secreted by the anterior pituitary. Its therapeutic use is still quite limited, while it is important for diagnostic applications. A different importance has been attributed to its analogs/antagonists, whose anti-proliferative activity especially concerns breast and prostate cancer. Two hPRL antagonists (S179D-hPRL and G129R-hPRL), discovered by two leading laboratories in the US and in France, have been synthesized in our laboratory for the first time in their authentic form. Giving thus continuity to the studies carried out in collaboration with the University of California at Riverside, where S179D-hPRL has been first studied and synthesized, *in vivo* antitumor and anti-angiogenic activities have been demonstrated, together with a particular conformational change due to an increased positive surface charge that activates different signaling molecules. In a few words this is what makes the difference between hPRL and its antagonist (Biochemistry, 2009).
Another study has focused directly hPRL production in *E.coli*, where expression levels had always been extremely low. This problem was resolved by carrying out activation at a lower temperature (37° C instead of 42° C), thus using the lambda PL promoter in a constitutive way, without its repressor. This way an approximately 30-fold higher expression was obtained, showing also that the bacterial cytoplasmic environment is apparently very harmful to hPRL stability, especially at temperatures > 37° C (Journal of Biotechnology, 2008). Besides in bacteria, hPRL synthesis had been also increased (~2-fold) in CHO cells, by the addition of an appropriate amount of sodium butyrate (1mM) to the culture medium, a method that we are now considering also for application to other hormones, as for example hTSH (Journal of Biomedicine and Biotechnology, 2010). Another type of addition has been also studied and experimented for CHO cell culture medium: cycloheximide. This compound has the property of slowing the protein synthesis, thereby extending the time available for the glycosylation machinery. As a result glycosylation site occupancy is also increased. The results obtained have been quite rewarding, with an increase of ~4-fold in the concentration of glycosylated prolactin (G-hPRL) and of ~7-fold in the glycosylated versus non-glycosylated hPRL (NG-hPRL) concentration ratio (Journal of Biotechnology, 2010). This way it will be easier to obtain higher amounts of this isoform and better study its physiological role, which is at present still unknown. Finally, a more clinical research was also carried out, in collaboration with the Neuroendocrine Unit of the FMUSP/São Paulo, in patients affected by systemic lupus erythematosus (SLE) hyperprolactinemia. The results indicated that lymphocytic hPRL does not contribute to hyperprolacinemia of SLE, supporting a pituitary origin for hPRL hypersecretion (Clinical and Experimental Rheumatology, 2010).

### 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 present study has been developed by the group focusing three interlinked aspects. The objective of the first one (cytogenetic aspect) consists on a

comparative study of the effects of different radiation types and radionuclides of medical interest ($^{153}$Sm, $^{177}$Lu, $^{131}$I), in human and rodent cells, by cytogenetic and biochemical techniques. Radiation damage to DNA and repair capacity in cell lines originating from breast cancer were also analyzed (International Journal of Low Radiation, 2009). A collaboration with the Parasitology and Malacology Laboratory of Butantan Institute has been developed for the evaluation of the genotoxic effect of environmental mutagens, using freshwater snail *Biomphalaria glabrata*, irradiated with $^{60}$Co (Mutation Research, 2008). The second aspect of our study consists in the establishment of dose-response curves for different types of radiations (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 and for the β radiation of $^{90}$Sr have already been establishment by chromosome aberration, micronucleus and comet assays. More recently, curves for fission neutrons produced in the Reactor R1 of IPEN-CNEN/SP were obtained in collaboration with the "Centro de Engenharia Nuclear" (INAC 2009). 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, with or without use of recombinant human thyrotropin (Thyrogen$^{R}$ or rhTSH), that is being carried out in collaboration with the Nuclear Medicine Center of FMUSP. This study was approved by FAPESP and new equipments were granted, among them a modern optic microscope Nikon Eclipse 80i, with image system. A previous Project, based on animal model, also granted by FAPESP, had already been concluded, the related results having been published (Radiation Environmental Biophysics, 2008). Finally, a study about the radiomodificator effect of propolis in normal and human prostate cancer cells is in progress, having already published data on the "Protective effect of propolis in radiation induced chromosomal damage in Chinese hamster ovary cells (CHO-K1) irradiated with $^{60}$Co" (INAC 2009). The data obtained via micronucleus test showed a radioprotector effect on the induction of DNA damage and an anti-proliferative effect on tumor cells were demonstrated by cytotoxicity tests. These data indicate a potential promising use of propolis as a natural, nontoxic, effective substance for protection against genotoxic and cytotoxic damages, induced by ionizing radiation

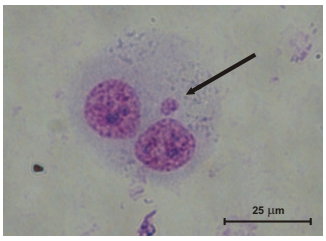


Figure 1. Binucleated cell with one micronucleus

**Protein radioiodination**

The Group of Hormones of IPEN 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 2001-2007, five more papers have been published, providing precious collaborations to other Brazilian research groups, in prestigious journals as well. During the period 2008-2010, object of the present Progress Report, four more papers have been published in Pancreas, European Journal of Pain, and Toxicon, in collaboration with the Departments of Pharmacology of Unicamp (Campinas - SP) and of the University of São Paulo (São Paulo - 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.

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## Biopharmaceuticals

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). Another approved FAPESP project about lyophilization process of biological tissues to make cardiac valves, includes IPEN, INCOR, UNICAMP and the Pharmaceutical Sciences Faculty of University of São Paulo. The biofunctionality of the bovine pericardium with endothelial cells has been tested.

### Recombinant proteins - refolding from inclusion bodies using high hydrostatic pressure

The bacteria *Escherichia coli* is the most efficient and cost-effective host for transgenic protein production for therapeutic as well as for research purposes. However, *E. coli* is often unable to fully process the recombinant foreign proteins during overexpression and thus misfolded proteins aggregate in bacteria cytoplasm. These aggregated proteins are known as inclusion bodies (IBs). Utilization of high hydrostatic pressure is a novel and robust method to disaggregate and refold proteins from inclusion bodies, by solubilization of the aggregates in mild conditions, maintaining the existing secondary and tertiary structure of the insoluble and mostly inactive proteins produced in bacteria. The Green Fluorescent Protein (GFP) is a monomeric protein that was initially extracted from the jellyfish *Aequorea Victoria*. It has the ability to convert ultraviolet light into a bright green fluorescence. The fact that the native structure must be present for emission of the characteristic fluorescence of this protein and the simplicity of monitoring the GFP bioactivity make this protein an excellent model system for refolding studies, enabling determination of an efficient protocol for protein refolding. In fact, contrary to what has been described in previous studies, we demonstrated that the pressure level that induced dissolution of eGFP IBs aggregates (2.4 kbar) was 7 times higher than the ideal conditions for refolding of this monomeric protein. By utilization of the Green Fluorescent Protein (GFP) IBs, as well as IBs of other proteins, as endostatin, QM, bothropstoxin 1 and cruzain, we demonstrated that high pressure can successfully convert insoluble protein from inclusion bodies to a preparation with native tertiary structure and fully biological activity. Endostatin can specifically inhibit endothelial cell proliferation and thus potently inhibit angiogenesis and tumor growth. QM or ribosomal protein L10 is originally identified as a tumor suppressor protein.

### 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. Although the exact extra-ribosomal functions of QM are not yet fully understood, it 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.

### Biological effects of ionizing radiation

Ionizing radiation, in aqueous solution, produces several highly reactive species. The most important are hydroxyl radical and hydrated electron. Theses 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 intravital microscopy; native crotamine causes a time-dependent vasoconstriction that suffers inversion with irradiated toxin (2 kGy) leading to the vasodilatation. Structural analysis suggested alteration in tertiary structure, keeping the primary structure unbroken.

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## Animal Laboratory Division

The Animal Laboratory Division is a facility having 1.040 $m^2$ 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.

Figure 2. 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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## Program Team

### Research Staff

Dr. Carlos Roberto Jorge Soares; Dr. Cibele Nunes Peroni; Dr. Daniel Perez Vieira; Dr. João Ezequiel de Oliveira; Dr. Kayo Okazaki; Dr. Ligia Ely Morganti Ferreira Dias; Dr. Maria Aparecida Pires Camillo; Dr. Maria Helena Bellini Marumo; Dr. Nanci do Nascimento; Dr. Paolo Bartolini; Dr. Patrick Jack Spencer; Dr. Regina Affonso; MSc. Miriam Fussae Suzuki; Tech. Antonio Carlos Junqueira; Tech. Cicero Florencio dos Santos; Tech. Dirce Pinto; Tech. Eduardo de Moura; Tech. Genivalda Pereira dos Santos; Tech. José Longino Ramos; Tech. José Maria de Souza; Tech. Manoel Calixto Lopes da Silva; Tech. Quitéria Pereira de Matos da Silva; Tech. Rosangela do Rocio Arkaten; Arlete Valéria de Souza Correia; Luis Efrain T. Miranda; Rute Marlene Batista; Sueli Tavares Venâncio.

### Graduate Students

Andrea Glezer; Andréia dos S. Silva; Beatriz Elaine de Almeida; Camila M. Yonamine; Claudia Regina Cecchi; Cristiane Moreira de Carvalho; Danielle Rodrigues; Diane Belchior Paraíba; Eliza Higuti; Eric K. Martins Ueda; Fabiana Medeiros da Silva; Fernanda S. Arthuso; Flávia Silva Valgode; Franco C. Bonetti; Geysa Spigoti Santos; Janaína B. Alves; José Alberto A. Silva; Karina Corleto de Oliveira; Larissa Pereira Miranda; Lucélia de A. Campos; Marco A. Júnior; Murilo Casare Silva; Natália M. Vallejo; Nelio Alessandro de Jesus Oliveira; Priscila Caproni; Renan F. Loureiro; Renata Damiani; Rodrigo C. Mosca; Rosa M. Chura-Chambi; Rosa M. Szarota; Samanta Etel Triger Borborema; Susana Rocha Heller; Taís Lima de Oliveira; Tatiana Franco da Cunha; Thiago França Malpighi Santos; Vanessa Borelli; Vicent Louis Viala.

### Undergraduate Students

Bruna Silvério; Danielle Gordilho Fernandes; Débora C. N. Salles; Diego J. O. Y. Belato; Edilson de O. Dantas; Emily Iusuti Lee; Fernanda B. Calvo; Flávia Calil Ambiel; Joseane F. Alves; Juliana L. Hashizume; Milena M. Leinmüller; Priscila Harumi Hara; Rodolfo G. Covre; Tatiana M. de Arruda; Vanessa Danielle Magalhães.

### Co-Workers

Dr. Alvaro A. Alencar de Queiroz; Dr. Alvaro R. B. Prieto da Silva; Dr. Andrea C. D. Rodas; Dr. Herbert Rodrigues Goulart; Dr. Luiz Augusto Corrêa Passos; Dr. Márcia Augusta da Silva; Dr. Maria Esther Ricci da Silva; Dr. Maria Teresa C. P. Ribela; Dr. Olga Zazuco Higa; Dr. Tetsuo Yamane; Dr. Vera Maura F. de Lima; MSc. Adriana Natsue Ozaki

## Honor Mention and Awards

The Brazilian Society of Science in Laboratory Animals (SBCAL) honored the work "Influence of ionizing radiation on well being 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, 2009.