Indigenous Production of Bovine/Bubaline Reproductive Hormones

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Abstract: The research work of our laboratory on buffalo pituitary hormones is summarized here in the context of MOET programme of our country. All the anterior pituitary protein hormones of this species (i.e. Follicle stimulating hormone (FSH), Growth Hormone (GH) and others) have been purified from freshly frozen pituitaries. These hormones have been extensively characterized with regard to physico chemical, immunochemical and biological features. We have also produced buffalo Prolactin (PRL), Luteinizing Hormone (LH), FSH and Thyroid Stimulating Hormone (TSH) by recombinant DNA techniques.

Keywords: Buffalo, pituitary hormones, superovulation, embryo transfer technology.

INTRODUCTION

Modern Science, as we understand today is of European origin. Post-renaissance science is what is termed as modern science. It spread to other countries through colonial rule. Science has to be practiced through scientific method. Moreover fundamental science is global and competitive. Application oriented science is necessary to solve national problems. When embryo transfer technology was proposed, in eighties, as one of the national technology missions to achieve 'Herd improvement by MOET', buffalo was the identified national target animal. Being application oriented work, a number of groups took up this work. NDRI, Karnal and NDDB, Anand were the most prominent among the groups which were later expanded to cover other animal groups. A large number of publications resulted from our country. In terms of success it was not very impressive. Success was visible only in the case of cross bred cows, especially in Kerala, but using Artificial Insemination and not Multiple Ovulation and Embryo Transfer Technology. More than 100 crores of rupees were invested in the form of infrastructure development and project running costs. In the case of buffaloes, a number of problems were identified and reported as the cause for the relatively unimpressive success [1]. One of the most important problems among others was the absence of indigenously produced homologous hormones and their specific antibodies. The work related to MOET involved many techniques and methods related to endocrinology, reproductive physiology, immunology, biochemistry and molecular biology. Being a first experience of its type, there were no trained manpower to begin with and so capacity building itself was a major task leave alone succeeding in establishing the technology.

Our laboratory initiated, in 1981, a long range programme of understanding buffalo endocrinology from a biochemical perspective. As part of the essential requirement for an independent career, our laboratory identified the first goal of becoming self-sufficient in buffalo hormones and specific antibodies [3] to enable us and other groups to undertake the more important project of understanding the biology part. We had no goal of participating in the MOET programme set for us. We restricted ourselves to basic science of hormones. It is only in 1987, in a meeting convened by Professors GP Talwar and PN Bhat [2] that buffalo MOET project became a national mission with DBT funds being distributed. As decided by the elders, the funds were initially earmarked for direct ET work on buffaloes to be executed by the different veterinary groups. This was what we thought so because our laboratory did not receive any funds. By then we had published few papers on the purification and characterization of buffalo pituitary hormones. A couple of them were in fact major discoveries in the field of protein hormones. In 1993, we were asked to submit a project on production of buffalo pituitary protein hormones concerned with reproduction. We did and the project was sanctioned in 1995. We completed the project and the result was that from an input of Rs 35 lakhs of rupees we could produce hormones and antibodies worth 50 million dollars going by the market prices for such products (from Sigma Catalogue). For us it resulted in few more publications. By 2000, every body in the country realized all the problems preventing successful MOET programme in buffaloes. In stage two, once again, the nation focused on applied aspects in a hurry to succeed in the technology. Now the new

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problem raised by our work was that the biochemical procedures developed and adopted by our laboratory gave unsatisfactorily low yield of FSH, the most important hormone for superovulation induction. The field workers added their own list of priority problems which was that though another hormone which is more biologically active than pituitary FSH i.e.PMSG (pregnant mare serum gonadotropins) can be used as a substitute it also led to either hemorrhagic follicles due to over stimulation or antibodies to PMSG. Both would naturally prevent reuse of the she-buffalo the next month. The division of labor between the egg donor mother and the surrogate mother which carries the implanted blastocyst was the key feature of this ET technology for rapid herd improvement. The solution to this vexed problem was use not biochemically isolated buffalo hormones but buffalo pituitary hormones made by rDNA technology. Once again the nation found itself in a fix.

There was no turnkey technology to make recombinant hormones of buffalo origin in the country or outside the country. The most important lesson we all learnt was the technology that a nation can practice is solely dependent upon its level of competence in the basic science related to that technology. Without understanding the basic endocrinology of buffalo, we jumped to ET project. Similarly without expert molecular biologists that can use rDNA technology to clone, express biologically active (*in vivo*) recombinant protein hormones, nobody could use such homologous hormones as nobody had them. Therefore the veterinary groups were trying alternate technologies to the use of FSH/PMSG. The ovsynch protocol and others were standardized. Not only that, few groups of workers started using abattoir collected immature follicles and tried to grow and differentiate them into Graafian follicles. In other words *in vitro* oocyte maturation and *in vitro* fertilization were also studied extensively and intensively. Once again many, reasonably good papers were published but these did not help 'Herd Improvement' on a scale which was originally envisaged. Critical analysis of these papers reveals that most of the protocols were based on such protocols which had been demonstrated in other species. There was no new logic for designing these experiments. The fundamental problem here is that we, as a group of scientists, did not invest time and work to understand the hormonal regulation of follicular maturation, ovulation, and oocyte maturation in buffaloes as opposed to in other species. We did not look for differences and for those factors which are species specific. The application oriented scientists were frustrated as neither biochemically purified hormones, synthetic peptide hormones like GnRH nor recombinant hormones of buffalo origin were available in large quantities, as the basic scientific work was never a priority for the funding agencies. The basic scientists, meanwhile, were identifying and studying more fundamental research problems not just on buffalo hormones but even PMSG. PMSG is LH like in the mare but FSH like in heterologous animals. We need to understand the basic biochemistry of this hormone to knock-out its LH like activity but retain FSH like activity. That would solve the problem of hemorrhagic follicles. Once again, our laboratory was asked, in a meeting at Karnal in 2006, to apply for the newly formulated NAIP programme in collaboration with veterinary groups to contribute to innovative solutions to veterinary problems. Our laboratory applied for the project support in collaboration with the National Research Center on Equines at Hissar, India. The story of 1987 was repeated. We did not get the financial support as it was not a high priority project. We are continuing to do our basic work on buffalo and pregnant mare hormones, whatever be its worth for application. The results of our 30 years of fundamental research work are described below. We are as frustrated as the veterinary groups. We might have published good papers but there has not been a demonstrated application of our products.

SUMMARY OF RELEVANT FUNDAMENTAL WORK ON BUFFALO HORMONES FROM OUR LABORATORY

The five most important pituitary protein hormones (FSH, LH, TSH, GH and PRL) which have roles to play in reproductive processes were purified from freshly frozen abattoir collected pituitaries and characterized [4-8]. Polyclonal and monoclonal antibodies to these hormones were raised and characterized and used to develop RIAs and ELISAs. Some of these have been applied to field samples also [9-10]. We were rewarded with some basic discoveries during these efforts [4, 11-15]. Few remarks on these findings should be relevant here.

The procedures were, all except one, biochemical in nature and were intelligent modifications of the then existing protocols which were developed for sheep pituitary hormones. Recently we demonstrated that textile dyes like Cibacron Blue, can be utilized to obtain pure preparations of LH in a 'one-step purification method' [16]. A major discovery in this work was that

the so called 'acid insoluble pellet' in the Papkoff protocol [17] which was routinely thrown into the sink by groups all over the world was shown by us as a richest source of Prolactin and Growth Hormone [18]. The preparations, though homogeneous as far as hormonal activity is concerned, exhibited 'Structural Micro heterogeneity'. We had initiated another major long term research programme to understand the physiological and evolutionary significance of this phenomenon of micro heterogeneity based on two hypotheses which are being investigated. One, that the microheterogeneous forms have different potencies in a given bioassay during ontogeny and two, that the microheterogeneous isoforms have different biological roles during phylogeny. We have data to substantiate this to some limited extent. For example, the glycosylated prolactin and the acidic prolactin have different potencies (ED50 values) in the Nb2 rat lymphoma cell line based bioassay when compared with the non-glycosylated and basic isoforms of buffalo prolactin [6].

Another feature which is noteworthy in these findings is that the yield of potent hormone per pituitary or kilogram of frozen pituitaries differs from hormone to hormone. While it is 3-4 mg in the case of FSH and TSH, it is 250 mg in the case of LH, 500-800 mg in the case of prolactin and 2-3 grams in the case of GH. In fact we later realized that the protocols were inefficient. We made modifications to substantially cut down the losses in the side fractions. This resulted in dramatic improvement in yields especially of GH from 2-3 grams to 19 grams per kilogram of pituitaries! [19]. In recent times we made another major discovery in the case of prolactin. The naturally occurring lower size isoforms of buffalo prolactin were shown to have anti-angiogenic activity in three bioassay systems like the CAM assay, wound healing assay and cell migration assay [20]. Not only that, we have demonstrated this activity in peptide fragments derived from Prolactin by the use of Cathepsin D [15]. Recombinant buffalo prolactin has been produced and characterized [24]. Further we located a peptide sequence within the sequence of both prolactin and Growth hormone, got the sequence synthesized and the synthetic peptide was active in these *in vitro* bioassays in picograms. The potential of these peptides as anti-cancer drugs need not be over emphasized [25]. Another interesting finding we have is that rat ovary responds to buffalo LH and hCG better than buffalo ovary *in vitro* in terms of cAMP levels. Not only the basal levels of cAMP in buffalo ovaries are less than that in rat ovaries, the response of buffalo ovaries to LH *in vitro* in terms of fold stimulation and absolute values of cAMP are muted when compared to that in the rat [21]. Further, the thyrotrophic effect of buffalo LH appeared to be better than that of buffalo TSH and hCG *in vitro* when thyroid fragments are incubated with hormones and cAMP response was measured. These unpublished results have reiterated the well known finding of veterinary groups that buffalo does not respond well to exogenously administered gonadotropins. Textbooks say that thyroid hormones enable other hormones to act on their respective target tissues [22]. Published work from veterinary and other groups lead us to believe that thyroid hormone levels (T4 and T3) in buffaloes are normal. While we are not saying that buffalo has a hypothyroid status, we believe that the interaction of Thyroid hormone and gonadotropins in the buffalo could be defective. Hormone levels do not always reflect hormonal response intensity.

LESSONS LEARNT AND SUGGESTED FUTURE COURSE OF ACTION

We believe that the homologous buffalo hormones should be fully evaluated for their *in vitro* and more important *in vivo* potencies in buffaloes. Further, long acting versions of recombinant FSH should be produced and tested for efficacy in inducing superovulation in buffaloes. Further, LH activity free PMSG should be produced by structure-function relationship studies followed by application of recombinant DNA techniques. Studies should be undertaken to understand the dynamics of and mechanisms underlying buffalo oocyte maturation including hormonal effects using homologous hormones. Studies should be initiated to assess quantitavely the gonadotropin and thyroid hormone receptor(s) status in buffalo gonads. Studies should be initiated in augmenting the buffalo ovarian response to administered gonadotropins by either dietary supplementation or through parentaral administration of permissive hormones. The interaction of prolactin action vis-à-vis gonadotropins action should be studied. We had demonstrated, long back, that prolactin can inhibit LHRH action on rat pituitaries *in vivo* in the pregnant lactating rat model under low intensity of suckling [23]. We have also observed that prolactin antagonizes LH and FSH action in the rat. Not only that the synthetic peptide, obtained based on the internal sequence of prolactin was found to increase ovarian ascorbic acid- an action which is opposite of that of LH on ovaries. Large scale production of recombinant buffalo FSH expressed in mammalian cells, which give

full complement of oligosaccharide to the protein, or in engineered yeast expression system to enable it to put glucosamine and sialic acid onto the recombinant hormone should be undertaken to ensure supply of buffalo hormones for veterinary groups.

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