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BARD

FINAL REPORT

PROJECT NO. US-2642-95

**Increasing Mammary Protein Synthesis
through Endocrine and Nutritional Signals**

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Milk, insulin, insulin-like growth factor, branched chain amino acids, starch, lipid

Abbreviations used in the report, in alphabetical order: AA, amino acids; AV, arteriovenous; ECM, extracellular matrix; IGF, insulin-like growth factor; NSC, nonstructural carbohydrates; RBC, red blood cell

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Abstract

Objectives

1. To determine endocrine factors that regulate the partitioning of amino acids by the mammary gland.
2. To evaluate dietary flow and supply of energy and amino acids and their effects on milk protein synthesis and endocrine status.
3. To use primary cultures of cow mammary epithelial cells to examine the role of specific factors on the rates and pattern of milk protein synthesis.

Milk protein is an increasingly valuable component of milk but little is known regarding the specific hormonal and nutritional factors controlling milk protein synthesis. The research conducted for this project has determined that milk protein synthesis has the potential to be enhanced much greater than previously believed. Increases of over 25% in milk protein percent and yield were detected in studies utilizing abomasal infusion of casein and a hyperinsulinemic-euglycemic clamp. Thus, it appears that insulin, either directly or indirectly, can elicit a substantial increase in milk protein synthesis if additional amino acids are supplied. For additional amino acids, casein provided the best response even though substantial decreases in branched chain amino acids occur when the insulin clamp is utilized. Branched chain amino acids alone are incapable of supporting the enhanced milk protein output. The mammary gland can vary both blood flow and extraction efficiency of amino acids to support protein synthesis.

A mammary culture system was used to demonstrate specific endocrine effects on milk protein synthesis. Insulin-like growth factor-I when substituted for insulin was able to enhance casein and α -lactalbumin mRNA. This suggests that insulin is an indirect regulator of milk protein synthesis working through the IGF system to control mammary production of casein and α -lactalbumin.

Principal component analysis determined that carbohydrate had the greatest effect on milk protein yield with protein supply only having minor effects. Work in cattle determined that the site of digestion of starch did not affect milk composition alone but the degradability of starch and protein in the rumen can interact to alter milk yield. Cows fed diets with a high degree of rumen undegradability failed to specifically enhance milk protein but produced greater milk yield with similar composition.

The mammary gland has an amazing ability to produce protein of great value. Research conducted here has demonstrated the unprecedented potential of the metabolic machinery in the mammary gland. Insulin, probably signaling the mammary gland through the IGF system is a key regulator that must be combined with adequate nutrition in order for maximum response.

Achievements

The mammary gland has a significant ability to produce milk protein. The potential for production is much greater than previously believed. Provision of insulin without hypoglycemia through the use of a hyperinsulinemic-euglycemic clamp resulted in unprecedented increases in milk protein content and milk yield. Further, the efficiency of amino acid use was dramatically improved.

Milk protein synthesis and mammary gland use of amino acids (AA) were not based on arterial concentrations of AA. The research conducted challenged a concept that was widely held and used as the basis for current computer models of milk protein synthesis. The mammary gland can vary both blood flow and extraction efficiency to support milk synthesis contrary to current computer models. Models that seek to tie AA supply with mammary gland utilization will have to be revised to incorporate the demonstrated responses by the mammary gland. Further, plasma was the primary pool for interorgan transportation of AA. This further invalidates current dogma and has major implications on deriving relationships between circulating AA and milk protein synthesis.

Principal component analysis using a large data set determined that carbohydrate is a major factor controlling milk protein yield while amino acid supply is only a minor regulator. However, application demonstrated that specific effects of carbohydrate on milk protein yield or percent were difficult. Provision of starch to the cow rather than to the rumen bacteria did not elicit any increase in milk protein synthesis. However, provision of both starch and protein to the cow increased milk yield with protein percent unaltered. Therefore, the cow did increase milk protein synthesis when additional carbohydrate was delivered to the cow plus additional protein was available to be utilized for milk protein synthesis; however, this resulted in greater milk yield as well so composition was unchanged.

The overall conclusion of the research was the confirmation that dietary energy primarily in the form of starch is capable of stimulating milk protein synthesis probably via an increase in circulating insulin or IGF-I. However, no increase in milk protein synthesis can occur until additional amino acids are present to provide the backbone of milk protein. The ability to specifically enhance milk protein content without increasing milk yield was not determined through the dietary manipulations examined in this project. Further research will have to explore that area.

The conclusions of the research conducted demonstrate the possibility for substantial improvement in milk protein yield. Application will take a few years before a consistent method to specifically enhance protein synthesis is determined. The estimated benefits are very difficult to determine at this time. A benefit to producers should be easy to recognize because many milk pricing schemes are moving to a protein-based system. Further, milk protein is a high quality protein for human consumption as well as for processing applications. The marketplace will drive the push for incentives for greater milk protein production as processors find other uses for milk protein. Other potential benefits of this research are a reduction in nitrogen excretion that would occur as cows become more efficient in the utilization of nitrogen consumed. Any reduction in nitrogen excretion would be beneficial to the environment. Additionally, transgenic animals used to make pharmaceutical proteins in milk may give much greater yields of these proteins if the animals are fed or treated in a way that enhances total milk protein synthesis. Clearly, a hyperinsulinemic-euglycemic clamp with additional amino acids can dramatically increase milk protein synthesis.

The cooperation in this project utilized the basic understanding of 2 researchers with the applied expertise of 2 other researchers. The collaborators

discussed possible application as the project was developed and results obtained.

The researchers met at every possible opportunity, and had frequent email conversations discussing experimental protocols and results.

Report

Background

Milk and other animal products are important as a food source contributing more than a third of the calories and between a third and all of other major nutrients in the U.S. food supply. For example, sixteen ounces of milk supplies 1/3 of the daily protein requirement of an adult and this protein provides an almost perfect pattern of amino acids for body needs. An increase in milk protein content is the focus of our efforts to improve the nutritional quality of milk. In addition to the aforementioned nutritional implications of increasing milk protein content, this would also lead to improved product quality and economic benefits. Milk protein content and quality have a great impact on cheese making and processing/handling of fluid milk and other manufactured milk products. Cheese and curd yield, fat globule emulsification, pH buffering and regulation of heat stability are among several factors positively affected by milk protein content. For a long time, milk price was determined substantially by fat content. Reduced consumer demand for milk fat and increased interest in cheese yields have increased the value of milk protein. Milk pricing schemes are changing to favor protein content. Thus, there are economic incentives in place for the producer to increase milk protein content. Further, under the situation of milk quota, such as in Israel, increasing milk protein content has substantial economic importance. Little is known about the regulation of milk protein synthesis. Some have hypothesized that the correct amount or pattern of amino acids is the key to the regulation of milk protein synthesis. Others have observed that dietary energy is correlated with milk protein content. The objectives of this research centered around determining the hormonal and nutritional factors important in the regulation of milk protein synthesis.

Methodologies and materials

Objective 1

Lactating Holstein cows were used in a split-plot design within a cross-over

experiment. The cows were rumen fistulated for infusion of water, casein or branched chain AA into the abomasum. Cows were fed every 4 h to ensure steady state flow of nutrients. Periods were 14 d with a 7 d rest period to prevent carryover effects. The first experiment evaluated the effects of abomasal infusion of casein or branched chain AA on milk protein synthesis without any endocrine treatments. Interactions of nutrient supply and insulin treatment were tested in a series of experiments. One experiment tested the effects of abomasal infusion of branched chain AA versus water. A second experiment tested the effect of water versus casein plus branched chain AA. Additionally, a third experiment evaluated the interaction of daily injections (40 mg) of bovine somatotropin and insulin. Baseline milk production and composition were measured on samples taken on d 8-10 of each period. On d 11-14 of each period, cows were subjected to a hyperinsulinemic-euglycemic clamp in which circulating concentrations of insulin were raised approximately 4-fold. Blood glucose was maintained by infusion of glucose. Insulin-stimulated changes in milk production and composition on d 14 of each period were compared to baseline values to determine the effects of insulin. Milk production and composition were determined on all milk samples. Blood samples were taken every 4 h on d 8-14 of each period and analyzed for insulin, urea nitrogen, AA, IGF-I and IGF binding proteins. Mammary uptake of metabolites was evaluated in one study using AV methodology.

A different examination of the endocrine control of milk protein synthesis utilized intramammary infusion of insulin or a modified IGF-I that did not bind to IGF binding proteins (LR³IGF-I). Hormones were infused into one udder half after each milking as a way to provide the tissue with possible endocrine mediators.

Objective 2

Cows in mid-lactation were fitted with ruminal and abomasal cannulas. Three of the cows were fitted also with ileal cannulas. Cows were fitted with blood sampling catheters that were placed in the costo-abdominal artery. Venal samples were taken from a temporary catheter inserted 1-d before commencement of blood sampling. This set up enabled measuring of metabolite AV differences across the

mammary gland. Cows were used in a 4 x 4 Latin square experimental design. All cows were fed a basal diet ad libitum with free access to water. Feed intake was recorded daily. Treatments were infusion of: 1) 1.5 kg of water in the rumen, 2) 1.5 kg of corn starch (Galam, Israel) into the rumen or to the 3) abomasum, or 4) 0.5 kg of corn oil (Milomor, Israel) in the abomasum. Each period of the experiment consisted of 14 d. Ten d were for adaptation followed by 4 d of sample collection (digesta, blood and milk). Chromium-mordant neutral detergent fiber was used as a marker to determine digesta outflow. Daily marker dose was applied in two equal portion into the rumen cannula. Ruminal, abomasal, ileal and fecal grab samples were taken during 11th to 13th sampling days in 2h intervals. Digesta flows of crude protein, neutral detergent fiber and non-structural carbohydrates (NSC) was determined. Ruminal ammonia N was determined. Blood samples were withdrawn simultaneously from the mammary vein and cost-abdominal artery on day 14th on each hour from 8 to 14h, and were transferred into heparinized vacutainers. Plasma was analyzed for urea, AA, glucose, insulin and IGF-I.

Thirty-six cows were housed in loose covered pens with adjacent yards. Cows were blocked into three equal groups according to parity, date of parturition, body condition score and milk yield during the first 150 d of the previous lactation. The 3 treatments were: A) a diet containing low level of rumen undegradable protein and a low level of rumen undegradable starch, B) a diet containing a low level of rumen undegradable protein and high level of rumen undegradable starch, and C) a diet containing a high level of rumen undegradable protein and a high level of rumen undegradable starch. Assigned values for ruminal undegradable protein and NSC of feeds were based on in situ. All feeds were mixed and fed once daily. The amount of feed offered was 110% of the previous intake. Cows were milked 3x/d (at 6, 14 and 20 h) and milk yield was recorded daily. Every 2 wk, milk from three consecutive milking was sampled and milk composition was determined and analyzed for fat, protein, and lactose content. Fecal grab samples were collected to evaluate digestibility and blood was collected for hormonal and metabolite analysis.

Nutritional factors that affect milk protein were analyzed from 93 dairy nutrition research studies from 1988 to 1997. Missing nutritional data were calculated using the Spartan Ration Evaluator/Balancer for Dairy Cattle so that values for crude protein and acid detergent fiber from Spartan were within 2.5% of the values reported in the research studies. This provided 389 observations with 3295 cow periods from cows producing more than 25 kg milk and less than 120 days in milk. Principal component analysis and regression were the statistical procedures used to analyze the data. Principal component analysis was used to identify a list of independent non-correlated variables that explained the greatest amount of the variation among the independent variables.

Objective 3

Bovine mammary epithelial cells were cultured for 8-10 d on floating collagen gels in the presence of different combinations of insulin, prolactin, cortisol, IGF-I and bovine somatotropin. Northern blot analysis of cells was conducted to evaluate treatment effects on mRNA expression of milk proteins. Additionally, the expression of extracellular matrix (ECM) proteins was examined.

Results and Discussion

Objective 1

The initial study investigated the separate and combined effects of branched chain AA and casein on milk protein concentration and yield. Cows were abomasally infused with water, branched chain AA (150 g/d), casein (600 g/d), or branched chain AA plus casein (44 and 600 g/d, respectively). Neither daily dry matter intake (24.2 ± 0.4 kg/d) nor milk yield (32.9 ± 0.4 kg/d) was affected by any of the infusion treatments. Infusion of branched chain AA had no effect on any milk production parameters, despite a 50% increase in their plasma concentrations. Modest increases in milk protein concentration (0.1%) and milk protein yield (62 g/d) resulted from the infusion of casein or branched chain AA plus casein. True protein and whey protein concentrations in milk were also marginally increased by infusion of casein and branched chain AA plus casein, and infusion of branched chain AA, casein, or both elevated milk non-protein

nitrogen content. Thus, little benefit from supplementation with branched chain AA and only modest effects of casein on milk protein concentration and yield was detected in well-fed cows.

Examination of the effects of insulin on the regulation of milk protein synthesis was tested in well-fed cows, with or without additional AA. Abomasal infusions of either water or a mixture of casein (500 g/d) plus branched chained AA (88 g/d) were utilized. During the last 4 d of each period a hyperinsulinemic-euglycemic clamp was performed; insulin was infused at $1.0 \mu\text{g/kg BW}^{-1}\text{h}^{-1}$ to increase circulating levels 4-fold and euglycemia was maintained by infusion of glucose. During abomasal water infusion, the insulin clamp increased milk protein yields by 15% (+128 g/d); when combined with abomasal infusion of casein plus branched chain AA, milk protein yield was increased 25% (+213 g/d). These increases resulted from equivalent increases in milk protein concentration and milk yield. Analysis of specific milk nitrogen fractions indicated that concentrations of casein and whey proteins in milk were increased by insulin clamp treatments; however, there were no major changes in the relative proportions of individual casein and whey proteins. Plasma concentrations of essential AA were reduced (33%) during the insulin clamp treatments; effects were most dramatic for the branched chain amino acids (41%) and their keto acids (45%). Thus, results indicated that insulin either directly or indirectly plays a key role in regulating milk protein synthesis.

A second study determined whether supply of branched chain AA would be sufficient to support the enhanced milk protein synthesis observed. Abomasal infusion of water or branched chain AA (150 g/d) were utilized. Infusion of branched chain AA failed to alter milk protein concentration or yield in the presence or absence of exogenous insulin. A modest increase (4%) in milk protein concentration was detected irrespective of abomasal infusion. No changes in patterns of protein in milk were detected. Thus, additional essential AA in casein are required to support the enhanced milk protein production during an insulin clamp.

The interaction of bovine somatotropin and insulin were tested in 8 lactating cows infused abomasally with casein (600 g/d). Cows were given daily injections of somatotropin during one period and received no injections during the other period. Bovine somatotropin increased milk yield, milk protein yield, and concentrations of IGF-I in blood as documented by many other studies. However, insulin clamps failed to increase milk protein synthesis or IGF-I in plasma. No obvious reason was found to explain the lack of a response to the insulin clamp other than a pronounced depression (35%) in feed intake that occurred on d 1-2 after initiation of the clamp. The decrease in feed intake did occur in the other clamp studies but not to the extent observed in this study. Cows were producing well (>35 kg/d) and appeared in good health so diet appeared to be appropriate. Amount of glucose infusion necessary to maintain euglycemia was also similar to the other clamp studies indicating that the insulin infused was biologically active. We have no explanation for the lack of response even after significant discussions with many collaborators.

We also examined the uptake and net utilization of AA for milk protein synthesis with cows that were well fed in terms of metabolizable energy and protein supply and responded to an insulin clamp. Treatments were designed to give a wide range in arterial AA concentrations and milk protein yield, and involved hyperinsulinemic-euglycemic clamps and abomasal infusions of casein plus branched-chain AA. Arteriovenous (AV) differences of essential AA across the mammary gland were linearly related to their arterial concentrations, but more importantly, milk protein yield was not related to either AV difference or arterial concentration for any of the essential AA. Thus, neither arterial concentrations nor AV differences of essential AA provided reliable estimates of the adequacy of AA supply or of milk protein yield. Despite greatly reduced circulating concentrations of essential AA, the mammary gland was able to support the increased milk protein yields during insulin clamp treatments. The coordinated changes that allowed this involved increasing extraction efficiency of essential AA and mammary blood flow. During the insulin clamp treatments, there were also

shifts in the apparent mammary uptake to output ration of AA. These shifts resulted in the balance of total AA nitrogen and carbon being maintained above unity for all treatments. Overall, results provide further evidence of the adaptations by the mammary gland, which allow for an adequate nutrient supply.

To examine the contribution of plasma and red blood cells (RBC) to mammary AA supply, we measured AV differences during AA supplementation (abomasal infusion of casein plus branched chain AA) and endocrine manipulation (hyperinsulinemic-euglycemic clamp) in well-fed cows. Results demonstrated that plasma was the primary pool for interorgan transport of AA to the mammary gland.

The RBC made minimal contributions for most essential AA, except for small quantities of leucine, methionine and threonine; RBC accounted for 14% of the total mammary uptake of methionine and 5-7% for leucine and threonine. Overall, the interorgan supply of AA for the mammary gland was primarily by plasma and the AV differences for mammary gland uptake of essential AA from plasma and whole blood were highly correlated. The contribution of RBC to the supply and exchange of AA by the mammary gland was limited to only a few AA but may need to be considered to obtain a complete AA balance for the mammary gland.

The yields of casein and whey protein were increased by insulin clamp as were the concentrations of IGF-I in plasma and milk. The concentration of insulin in milk also increased during the insulin clamp and mimicked the temporal increase in yield of milk proteins. These data suggest that elevated circulating concentrations of insulin and IGF-I are potential candidates directly responsible for the increased yield of milk protein during the insulin clamp. Direct examination of hormonal effects on the mammary gland utilized a split-udder approach involving intramammary infusion of insulin and LR³IGF-I. However, intramammary infusion of insulin or LR³IGF-I in combination with supplementary protein supply had no positive effects on milk yield or the concentration of milk protein. Overall, data offer no support to a mechanism whereby insulin or LR³IGF-I directly influences milk protein production in the well-fed cow; however, because no response was observed we can not rule out the possibility that results may have been a

consequence of limitations of the intramammary infusion technique.

Objective 2

The effect of infusion of similar energy equivalents as starch to the rumen, or infusion of starch or oil to the abomasum was studied in 4 mid lactating cows; control were infused with water to the rumen. Infusions of starch into the rumen or infusion of starch or oil into the abomasum was associated with a decrease in voluntary feed intake. Overall energy intake was reduced in oil infused but not in starch infused cows. Nonstructural carbohydrate digestibility in the rumen was similar among treatments, but small intestine digestibility was higher in abomasally-infused starch. In these cows, 3.4 kg/d of NSC was apparently digested in the small intestine. Milk production was reduced in oil infused cows, but efficiency of milk energy and protein yield were unaffected by treatments. The plasma levels of glucose, glucose extraction rate, insulin and IGF-I concentration, rumen ammonia or plasma urea, arterial and mammary extraction rate of AA were similar among treatments. In conclusion, high amounts of starch could be digested in the rumen or the small intestine of dairy cattle. No metabolic advantage for increased supply of starch into the rumen or to the abomasum of mid-lactating dairy cows maintained on high concentrate diets and exhibiting a positive energy balance was evident.

The combined effect of feeding diets high in rumen undegradable starch and protein was studied in early-lactating cows. Feed intake was similar among diets. Total tract dry matter and neutral detergent fiber digestibility was higher in cows fed a high level of rumen undegradable protein and high level of rumen undegradable starch. Compared with low levels of rumen undegradable protein and starch, high levels delivered an extra 700 g/d of NSC and 250 g of intake crude protein to the small intestine. Plasma urea was lower in cows fed the high rumen undegradable protein and high rumen undegradable starch diet. In these cows milk energy output was higher than in the other treatments. It was concluded that additive nutritive effects could contribute to the improved milk production in cows fed the high rumen undegradable protein and high rumen

undegradable starch diet. Suggested mechanisms are improved NSC digestion in the small intestine, an improved intestinal crude protein flow, and reduced costs of urea synthesis.

Principal component analysis was used to identify a list of independent non-correlated variables that explained 75% of the variation among the independent variables. The best 4 principal components included 1) acid detergent fiber (ADF), neutral detergent fiber (NDF) and NSC, 2) animal and vegetable fat, 3) undegradable intake protein (UIP), and 4) degradable intake protein (DIP). The major principal component represents the carbohydrate fraction of the diet suggesting that dietary energy and not protein is the major determining factor in the regulation of milk protein yield. The best fitting, most parsimonious regression model was milk protein yield (kg/d) = $-1.755003 + 0.328613 \cdot \text{DIP} - 0.007862 \cdot \text{DIP}^2 - 0.051459 \cdot \text{NDF} + 0.000728 \cdot \text{NDF}^2 + 0.083281 \cdot \text{NSC} - 0.000568 \cdot \text{NSC}^2 - 0.003234 \cdot \text{DIP} \cdot \text{NSC}$. The results indicated a curvilinear decrease in milk protein yield as NDF increased. The DIP*NSC interaction revealed that when NSC was high, increasing DIP above the mean content in the database decreased milk protein yield, whereas at low NSC, increasing DIP increased milk protein yield.

Objective 3

Cultures of primary bovine mammary epithelial cells evaluated the effect of different culture conditions on the level of milk protein gene expression and synthesis of milk proteins in vitro. The lactogenic effect of various combinations of insulin, hydrocortisone, prolactin, IGF-I and bovine somatotropin as well as the effect of calf tail collagen and ECM isolated from lactating bovine mammary gland on milk protein expression and synthesis. In the tested culture conditions, IGF-I acted synergistically with insulin to increase milk protein gene expression in medium containing prolactin and hydrocortisone. Substitution of IGF-I for insulin in this medium resulted in elevated level of α -, β -, and κ -casein and α -lactalbumin mRNA. Somatotropin showed greater lactogenic effect compared to prolactin in the medium containing hydrocortisone and insulin. Thus, the results suggest that

both hormonal milieu and substratum for cell culture have dramatic impact on the level of cell differentiation, and, therefore, on the ability of cells to produce milk proteins.

Scientific implications

Overall, results from all studies provide valuable data on the relationships between AA supply and milk protein synthesis which can be immediately applied in computer models. Of particular significance, the results confirm the important role of the endocrine control in the regulation of milk protein synthesis and demonstrate that the mammary gland's capacity for milk protein synthesis is still not functioning at a maximum, even where cows are fully fed. Literature results indicate that where cows were fully fed to requirements so that neither metabolizable protein nor energy were limiting, the supply of extra AA alone gives only modest increases in milk protein yield (3.2%). Thus, simply providing the mammary gland with more AA in well-fed cows does not guarantee increased milk protein yields. In contrast, chronic elevation of circulating insulin caused a substantial stimulatory effect on milk protein yield, which was optimally expressed in the presence of additional AA (25%). Obviously, the key to application will be to develop a clear understanding of the endocrine regulation of milk protein synthesis.

Potential impact on agriculture, industry, environment, policy, legislation

Milk protein is an increasingly valuable part of milk. Pricing systems around the world are adopting methods to pay for this important milk component. Clearly, techniques to routinely produce higher protein milk will be economically beneficial to producers. Further, consumers will have greater access to a very high quality protein. In the United States, more milk is being utilized for cheese, a process that benefits from greater protein content. The environment would also benefit from the knowledge gained by this research, as more efficient milk protein production will reduce the amount of nitrogen being excreted by the cow.

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Patent list

None

Publication Summary (numbers)

	<u>Joint</u>	<u>US</u>	<u>Israeli</u>	<u>Total</u>
<u>Refereed (published, in press, accepted)</u>	0	7	0	7
<u>Submitted, in review, in preparation</u>	0	2	2	4
<u>Invited review papers</u>	0	0	0	0
<u>Book chapters</u>	0	0	0	0
<u>Books</u>	0	0	0	0
<u>Master theses</u>	0	2	0	2
<u>Ph.D. theses</u>	0	2	0	2
<u>Abstracts</u>	0	12	1	13
<u>Not refereed (proceedings, reports, etc.)</u>	0	4	0	4

Patent Summary (numbers)

	<u>Israeli inventor only</u>	<u>US inventor only</u>	<u>Joint IS/US inventors</u>	<u>Total</u>
<u>Submitted</u>				0
<u>Issued (allowed)</u>				0
<u>Licensed</u>				0

Cooperation Summary (numbers)

	<u>From US to Israel</u>	<u>From Israel to US</u>	<u>Together, elsewhere</u>	<u>Total</u>
<u>Visits/Meetings</u>	0	3	0	3
<u>Sabbaticals</u>	0	0	0	0

Postdoctorates	0	0	0	0
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Cooperation, briefly explain whether synergistic, complementary or supportive.

The investigators supported each other throughout the project. Questions about animal procedures and laboratory techniques were discussed before being used. Investigators met at the annual meeting of the American Dairy Science Association to discuss progress and hypotheses. Attempts were made for complementary efforts through sending samples from Israel to the United States. However, regulatory issues concerning the importation of bovine tissue samples inhibited this option.