

Effects of Gallbladder Removal on Cholecystokinin-Mediated Food Intake In Mice

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ABSTRACT

In the senior population a decrease in food consumption resulting in weight loss often occurs. In addition, gallbladder functional decline or removal (cholecystectomy) may occur with age. The hormone cholecystokinin (CCK) may be involved in age-induced anorexia and weight loss. Among CCK actions are stimulation of gallbladder contraction and central nervous system mediated satiation after food intake. This study investigated how cholecystectomy in mice affects food intake, body weight, and plasma CCK levels. A sham-operated control group (SHAM) of nine mice with their gallbladders intact was compared to the experimental group (GBX) of ten mice that underwent cholecystectomy. Body weight and food intake were measured and reported weekly. After ten weeks, plasma was collected for measurement of CCK levels by radioimmunoassay (RIA). There appeared to be trends toward increased body weight, decreased food intake, and increased fasting plasma CCK levels ($p < 0.072$) in the GBX group, but these trends will require further study.

INTRODUCTION

As people age, their food intake linearly decreases. This is due to both decreased metabolism and decreased physical activity(1). Thus, the elderly may experience a physiological form of anorexia that may or may not lead to weight loss. The decrease in metabolism may be due to hormonal alterations that occur with aging. Cholecystokinin (CCK) is one hormone whose secretion is altered with aging. CCK secretions may be altered due in part to delayed gastric emptying. Delayed gastric emptying tends to increase with age. Thus, decreased food intake along with stomach emptying delay is correlated with anorexia in aging (2).

The gallbladder is an organ that helps to increase the rate and efficiency of fat digestion in the small intestine. This is accomplished via bile emulsification of large fat globules into small micelles. Gallbladder removal may occur as a result of gallstones (precipitation of concentrated bile constituents) or gallbladder disease. The secretion of bile, which is produced in the liver and stored and concentrated in the gallbladder, is under the influence of CCK. CCK is released into the blood by duodenal enteroendocrine "I" cells in response to fat entering the small intestine. When CCK binds to its receptors (CCK-A receptors) on the gallbladder, the smooth muscle cells contract and bile is released. Emulsification allows pancreatic lipase, an enzyme secreted in response to CCK, to work more efficiently in digesting or breaking down triglycerides (1). Bile components reduce CCK release (negative feedback mechanism), and when the release of bile is reduced in the presence of fats, CCK secretion increases (3). With gallbladder removal there are less CCK-A receptors present, which may result in a decrease in the plasma CCK clearance.

Other actions of CCK binding to specific receptors include: stimulation of secretion of pancreatic enzymes and other hormones in the digestive process, relaxation of gastric smooth muscle, contraction of the pyloric sphincter leading to decreased gastric emptying rate, and relaxation of the sphincter of Oddi. CCK has also been shown to be a potent satiety signal. As a satiety signal, CCK affects the ventral medial hypothalamus, an important control center for feeding behavior. A second CCK hormone receptor is the CCK-B (brain origin), which is located in the pancreas and in the hypothalamic region of the brain. Both CCK-A and CCK-B receptors are either Gs or Gp protein linked (3). It has also been hypothesized that CCK may act as a neurotransmitter (neuropeptide) in the brain and enteric nervous system (4).

Together, gallbladder removal and decreased CCK plasma clearance may be interrelated in causing decreased food intake and weight loss in the elderly. As CCK is proposed to induce satiety, increased CCK levels reaching the brain may ultimately lead to anorexia. The purpose of this study was to determine whether removal of the gallbladder leads to fluctuations of CCK, and whether or not these fluctuations are related to anorexia. The project was carried out to determine if appetite suppression, weight loss (due to reduced food intake), and changes in plasma and brain CCK levels change follow gallbladder removal in mice.

MATERIALS AND METHODS

Animal care. Nineteen male adult age-matched Swiss mice (Harlan-Sprague Dawley, Madison, WI) were housed individually with a twelve hour light-dark cycle and fed standard rodent chow (Teklad, Harlan-Sprague Dawley, Madison, WI).

Surgery. Following a one week acclimation period, the mice were weighed, randomly assigned to, and underwent sham or gallbladder (GBX) surgeries under ketamine (80 mg/kg)/xylazine (20 mg/kg) anesthesia. For gallbladder removal, a 1-2 centimeter midline incision was made through the skin and abdominal muscles below the diaphragm. The gallbladder was removed by tying off the cystic duct with suture and cutting it at the base of the gallbladder. Muscles were sutured closed and the skin was closed with glue. The mice were then carefully monitored until awake. Following surgery, body weight and food intake were precisely measured and reported on a weekly basis. After ten weeks, the mice were killed by CO₂ inhalation followed by cardiac puncture for collection of plasma. The brain, liver sections, and the duodenum were removed and frozen in liquid nitrogen.

CCK Radioimmunoassay. Plasma CCK levels were assessed by radioimmunoassay (Immunodiagnostic Systems Limited, England). Following CCK and recovery control extractions (in 96% ethanol) and drying, 70 µl of standards, controls, and samples were loaded into their respective polystyrene tubes. One hundred and seventy-five µl of anti-CCK-8 was added to each tube, followed by vortexing. The tubes were then incubated for two days at 4 degrees Celsius. Following incubation, one hundred and seventy-five µl of ¹²⁵I-CCK-8 was added to all tubes. The tubes were vortexed and then incubated for another 4 days at 4 degrees Celsius. After four days, 35 µl of Double antibody solid phase was added to all tubes and the tubes were vortex-mixed. The tubes were incubated for 30 minutes at 4 degrees Celsius and then centrifuged for 15 minutes. The supernatant was decanted, and radioactivity was counted in standards, controls, supernatant, and pellet samples using a scintillation counter. Plasma CCK concentration levels were determined against a standard curve.

Statistics. Effects of surgical treatment on body weight, food intake, growth curve slopes, plasma CCK and were assessed by t-test. Differences between means were considered significant at the critical value $p < 0.05$.

RESULTS

It was found that the GBX exhibited lowered average food intake, but greater average weight gain and plasma CCK levels when compared to the SHAM group. Figures 1 and 2 represent average body weight and individual body weight measurements, respectively. Figures 3 and 4 represent average and individual food intake, respectively. Statistical analysis of CCK plasma levels (Figure 5) show a p-value of 0.0725 was obtained for CCK plasma levels (pmol/L).

Figure 1. Average body weight measurements.

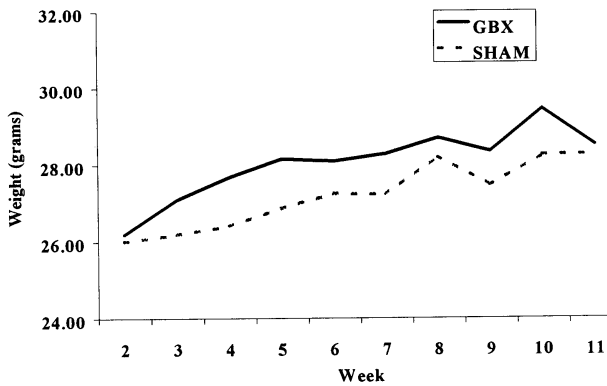


Figure 2. Individual body weight measurements.

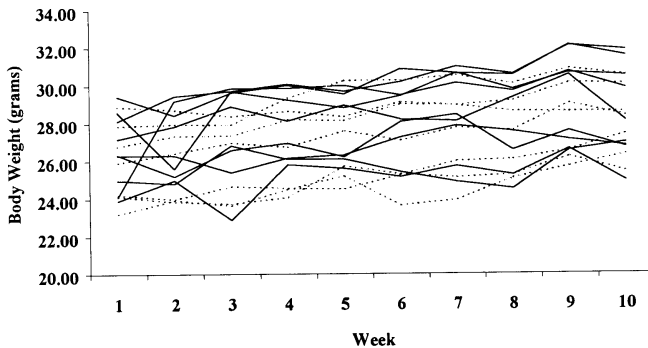


Figure 3. Average weekly food intake measurements.

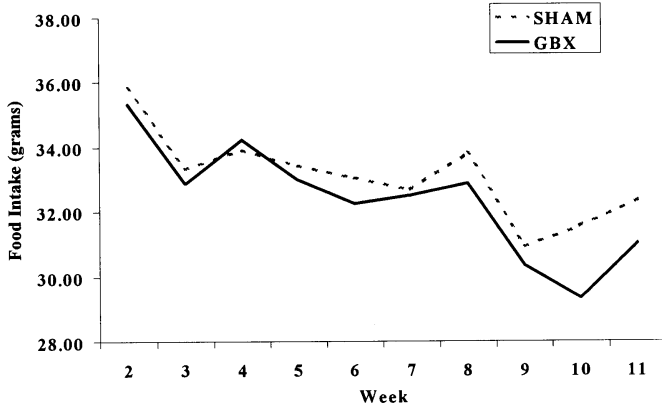


Figure 4. Individual weekly food intake measurements.

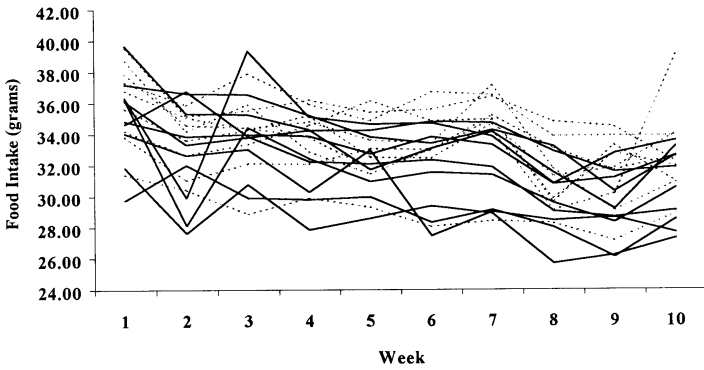
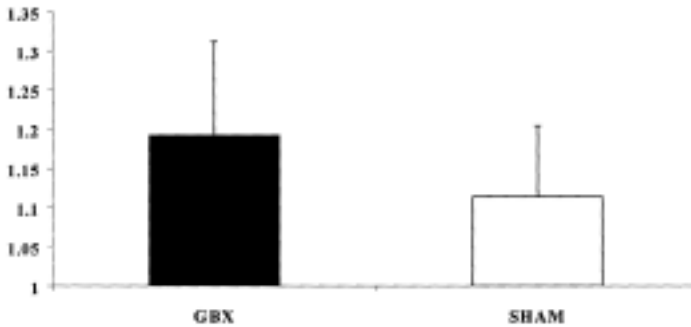


Figure 5. Plasma CCK levels. Values are expressed as means and standard deviations.



DISCUSSION

While trends in food intake and plasma CCK levels of the GBX group were towards the projected hypothesis (decreased food intake and increased plasma CCK levels), there were no significant mean differences. Therefore, the present data was inconclusive. Further studies need to be completed to determine if gallbladder removal leads to decreased appetite, decreased food intake, and ultimately weight loss.

Problems leading to inconclusive results were concentrated in the radioimmunoassay component of the project. The RIA used was designed to bind to human plasma CCK, not mouse plasma CCK. There was no mouse CCK assay on the market when this study was completed. Therefore, all recovery controls were performed with human plasma rather than mouse plasma. Since the recovery control did not come for a donor mouse, determination of the mouse accuracy of the assay was unattainable. The assay also required larger amounts of plasma than could have been recovered from the mice. Thus, the decreased initial plasma levels may have also affected the accuracy of the RIA, although the manufacturer of the assay agreed that it could be scaled back for mouse samples. A high speed refrigerated centrifuge specified for the assay was not available for use and therefore the samples were spun at a slightly lower speed and at room temperature. Due to the problems associated with the RIA, the confidence in the obtained values was decreased.

In conclusion, there was enough difference between GBX and SHAM groups in their food intake and plasma CCK levels to suggest further investigation, although the differences were not statically significant. A follow-up study is necessary to determine whether the observed differences may be significant.

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Location of Lymphocytes in the Bovine Mammary Gland

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ABSTRACT

Mastitis is an inflammation of the mammary gland, often due to bacterial infection. Farmers lose millions of dollars every year due to mastitis in cattle. Because of this problem, we are interested in the immunology of the bovine mammary gland. This study entails generating a broad picture of the location of the cells thought to be responsible for the specific immune response to foreign antigens in the mammary gland. It focuses on CD4, CD8 and $\gamma\delta$ T cells and B cells. To identify the location of these cells in the bovine mammary gland, tissue samples were collected from the right rear quarter of the mammary glands of six cattle. The tissue samples were taken from several sites vertically distributed from the teat end, along the teat canal and up into the upper gland and from several sites distributed laterally around the junction of the upper teat and the gland. Tissue blocks were snap frozen.

Monoclonal antibodies directed against CD4, CD8, $\gamma\delta$ T cell receptor and CD21 and immunohistochemical techniques were used to determine the distribution of T cell populations and B cells. Analysis of upper gland locations indicated a predominance of CD8 T cells, with fewer CD4 T cells. The teat area contained more B cells than the upper gland. Gamma delta T cells were rare throughout the gland. A monoclonal antibody specific for MHC class II stained many cells throughout the gland.

INTRODUCTION

Mastitis is one of the leading diseases in dairy cattle today. Mastitis is an inflammation of the mammary gland, which can cause abnormalities in the milk. A cow with mastitis can be identified when it is being milked, because the milk will either be chunky like cottage cheese or watery looking. In many cases the quarter that has mastitis will be swollen and hard. Mastitis is the result of bacteria infecting the mammary gland and the abnormality in the milk is due to the influx of macrophages, neutrophils and lymphocytes that are trying to kill off the bacteria. The National Mastitis Council reports that one of every three dairy cows contracts mastitis. The best way to treat mastitis is to give the cow antibiotics for 2-4 days. During the treatment and for several days after, the milk cannot be sold and is thrown away. In 1994, US farmers lost over \$2 billion due to mastitis (1).

The natural defense of the bovine mammary gland involves a variety of immune mechanisms with both non-specific and specific purposes. Lymphocytes are the cells responsible for the specific immune response to foreign antigens. These cells are responsible for the immunological memory that is necessary for a faster, stronger immune response when exposed to the same antigen at a later time. Vaccines target this part of the immune response by producing immunologic memory without causing an individual to have the full-blown dis-

ease. Vaccines are administered in a variety of ways and forms. For example, polio vaccine is given orally because it is known to generate memory lymphocytes in the area surrounding the gut. For a mastitis vaccine, it would be useful information to know what types of lymphocytes are present in the gland, and their distribution.

Milk has been used in studying the immunology of the mammary gland because it has been postulated that the cells in milk reflect the cells in the gland tissue. Out of the total population of cells in a cow's milk, 1-2% of them are lymphocytes indicating the presence of specific immune activity in the mammary gland (2). Even though milk has been valuable in studying the types of lymphocytes, which enter the mammary gland, it is very difficult to work with cells derived from milk in tissue culture due to the presence of bacteria and debris. Furthermore, milk may not reflect what is happening in the tissue. Therefore, it would be more accurate to study the cells in tissue samples using enzyme digestion to release the lymphocytes from the tissue. Because it is not clear exactly where lymphocytes are predominantly located in the mammary gland it would be valuable to determine the distribution of the lymphocytes in the mammary gland. Once the distribution of lymphocytes in the gland is known, areas rich in lymphocytes could be targeted for studies using enzyme digestion to release lymphocytes for further in vitro studies.

MATERIALS AND METHODS

Mammary glands. Cows were accessed through North Bend Processing, North Bend WI. Mammary glands from a total of six different cows were collected. History of the dairy cow was taken and cows were selected that did not have mastitis. The right rear quarter was taken from freshly slaughtered cows.

Frozen tissue sections. Within 4 hours of slaughter, the right rear quarter was trimmed to resemble a single slice up the center of the quarter. Fourteen cubes of tissue (10 x 10 x 5 mm) were taken throughout the slice (see figure 1), but mainly concentrating around the upper teat sphincter. The tissue cubes were placed in disposable specimen molds (Miles Inc. Elkhart, IN). Embedding medium (O.C.T. compound, Fisher Scientific, Pittsburg, PA) was put onto the tissue cube until the meniscus was above the surface of the mold. Water soaked cork pieces were placed onto mold to keep tissue section and medium in place. Sections were then snap frozen by placing them in 2-methyl butane immersed in liquid nitrogen for 20 seconds. The sections were then de-gassed by placing the sections on dry ice for 10 minutes. The sections were placed in sealed plastic bags and stored at -80°F. A cryostat was used for slicing the frozen tissue cubes into sections 6µm thick. Sections were collected onto glass microscope slides.

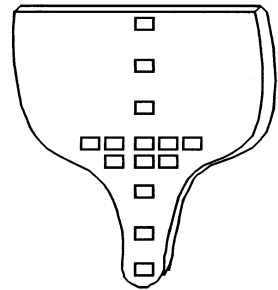


Figure 1. Section of right rear quarter of bovine mammary gland showing sites from which tissue cubes were collected

Staining. Slides with tissue sections were fixed in acetone for five minutes. After drying, a PAP pen was used to draw a wax circle around the tissue section to contain staining liquids. The slides were then incubated for 30 minutes with 100µl of 0.3% H₂O₂ at room temp to inhibit endogenous peroxidase activity. After rinsing for 5 minutes in PO₄ saline PH. 7.2

(Becton Dickinson Microbiology Systems Cockeysville, MD.) on a rocker, the slides were blocked with 100 μ l of 10% goat serum in saline and allowed to stand for 30 minutes. Excess serum was drained off and 100 μ l of primary antibody was added and incubated for 30 minutes. Table 1 lists the primary antibodies used to identify T and B cells. Another primary antibody, the monoclonal antibody UC-H9 was also used to analyze MHC class II expression in all tissue sections. After removing excess antibody, the slides were rinsed three times in PO₄ saline on a rocker for five minutes. Biotin-conjugated goat anti-mouse IgG (Zymed, South San Francisco, CA) diluted 1:1000 in 1% bovine serum in PO₄ saline was placed on the slides for 30 minutes. Slides were rinsed three times in saline on a rocker for five minutes. Horseradish peroxidase conjugated streptavidin (Zymed) diluted 1:400 in PO₄ saline was added and incubated for 30 minutes then rinsed three times in PO₄ saline for five minutes. Amino ethyl carbazole (AEC) working solution prepared from 1ml AEC stock, 9ml of Acetate buffer (pH 5.2) and 5 μ l of 30% H₂O₂ was filtered using a 0.22 micron syringe filter. The AEC working solution was added to slides and allowed to develop. Immersing the slides in distilled water stopped development. Slides were counterstained by dipping into Mayer hematoxylin for 45 to 60 seconds, rinsed in distilled water, then dipped in Scott solution for six seconds and rinsed in distilled water. The stained tissue sections were mounted in gel/mount (Biomedica Corp., Foster City, CA) medium and sealed with clear nail polish.

RESULTS

Tissue cubes were taken from 14 locations in the mammary gland as shown in figure 1. Sections of mammary gland tissue from each of the cubes from four cows were analyzed for tissue location and cell density of CD4+ T cells, CD8+ T cells, B cells and $\gamma\delta$ T cells and for expression of MHC class II.

The gland is arranged in a highly branched compound structure of tubuloalveoli lined with simple cuboidal epithelium. Groups of tubuloalveoli secretory units form lobules separated by intralobular connective tissue. Among the alveoli, loose connective tissue underlies the cuboidal epithelium. It was noticed that some of the cell types studied tended to localize in certain types of tissue (for example loose connective tissue versus intralobular connective tissue). It was also noticed that certain cell types were more numerous, depending on the general location in the mammary gland.

The CD4+ T cells were present, but in relatively few in numbers in the loose connective tissue, and were rare in the intralobular connective tissue throughout the gland. Very few CD4+ T cells were found in the epithelium surrounding alveoli.

The CD8+ T cell distribution was more varied, depending on the region of the gland. The dorsal and middle region of the gland contained some CD8+ T cells among the cuboidal epithelial cells lining of the alveoli. The loose connective tissue in these areas contained relatively high numbers of CD8+ T cells. Only a few CD8+ T cells were found in the intralobular connective tissue. The ventral or teat region of the gland contained small numbers of the CD8+ T cells scattered throughout the epidermis and grouped in pockets of loose connective tissue.

There were no $\gamma\delta$ T cells in the middle, and ventral or teat regions of the gland, but occasionally a few were found in the loose connective tissue in the most dorsal region of the gland.

The B cell numbers varied depending on the type of tissue and the region of the gland. Relatively few B cells were found in the loose connective tissue or epithelium surrounding

alveoli. The dorsal and middle regions contained numerous B cells in the intralobular connective tissue, while the ventral and teat regions were highly populated with B cells in the fibrous interstitial connective tissue.

All regions of the gland contained many MHC class II expressing cells both in the loose connective and in the intralobular connective tissue. MHC class II appeared to be expressed by blood vessel endothelial cells in the loose connective tissue throughout the gland.

DISCUSSION

The bovine mammary gland is a large highly structured organ composed of alveoli surrounded by secretory epithelium, supported by connective tissue. The cuboidal secretory epithelial cells secrete milk into the alveoli. The alveoli converge to ducts, which lead to a main duct opening through the teat. Bacteria are able to enter the gland through the teat canal causing mastitis.

Two T Lymphocyte populations in the mammary gland were studied: CD8+ and CD4+ T lymphocytes. CD4+ T lymphocytes, also known as T helper cells, release cytokines and detect antigens associated with MHC class II proteins where they present antigens brought into cells from the outside. This study showed that CD4+ T cells are present in uninfected mammary glands, although in fewer numbers compared to CD8+ T cells. This study also showed that MHC class II proteins are found throughout the gland. High expression of MHC II would allow for high levels of antigen presentation to T helper cells. Studies have shown that CD4+ T lymphocytes are increased in the milk after an infection of the mammary gland and become a greater percentage than the CD8+ T lymphocytes (6). This suggests that CD4+ T lymphocytes do play a role in the immune response to bacterial induced mastitis.

CD8+ T lymphocytes, also known as cytotoxic T cells, release cytokines and detect antigens associated with MHC class I proteins. MHC class I proteins can only present antigens produced in the cytoplasm of infected cells. T cytotoxic cells kill virus-infected cells and tumor cells. Our results indicated a greater concentration of CD8+ T lymphocytes than CD4+ cells in the upper portion of the uninfected mammary gland. This reflects the finding that CD8+ T cells outnumber CD4+ T cells in milk from healthy cows (6). This substantial population of resident CD8+ T cell may have some protective or regulatory function in the normal gland. Some reports suggest that the CD4+ T lymphocytes are inhibited by active CD8+ Lymphocytes (4). The greater number of CD8+ T lymphocytes in the mammary gland could perhaps decrease inflammation induced by T helper cells after a bacterial infection.

It has been shown that antibodies found in milk are produced locally within the mammary gland as well as in regional lymph nodes. This study confirmed the presence of resident B cells in the gland tissue (5). Since the bacteria enter the gland through the teat canal, it is speculated that a greater number of B cells will be found near the point source of infection, around the sphincter at the end of the teat canal. This was supported by our results of the higher concentration of B cells in the ventral portion of the gland.

Functions of the $\gamma\delta$ T lymphocytes are not established, but they are found in relatively high numbers underlying the epithelium in the intestinal tract and underlying the skin. Our results did not show a significant concentration of $\gamma\delta$ cells anywhere in the gland mammary, suggesting that these cells do not play a major role in local protection of the mammary gland.