Effects of limited food intake and vitamin C supplementation on pancreatic glucagon and insulin in guinea pigs

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The aim of this study was to investigate the effects of limited food intake (LFI) (24, 48 and 120 h) and a single i.p. dose of vitamin C supplementation (500 mg/kg) on serum glucose and C-peptide levels, and pancreatic insulin and glucagon levels in guinea pigs. The highest serum glucose levels were found after vitamin C supplementation plus LFI for 48 h (LFI 48). Serum C-peptide levels were not significantly affected by food limitation (LFI 24, LFI 48, or LFI 120) as compared with controls, but when vitamin C was supplemented, the C-peptide levels were moderately enhanced. Immunohistochemical findings on pancreatic islets showed increased staining intensity for both insulin and glucagon when vitamin C was supplemented. In addition, the alpha and beta cells were stimulated, particularly by vitamin C supplementation plus LFI 120. Based on these findings, vitamin C supplementation may have a beneficial effect on the alpha and beta cells.

Key words: Vitamin C, limited food intake, pancreas, C-peptide.

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The pancreas is a gland that has both endocrine and exocrine functions. Approximately 90% of the pancreas functions as an exocrine gland; the remainder comprises the endocrine pancreas, blood vessels, and interstitial fluid. In humans, the islets of Langerhans make up 1-2% of the pancreatic mass. There are approximately one million islets of Langerhans, each containing about 2,500 cells. Beta cells comprise 65% of the islet cells and secrete insulin, alpha cells comprise 20% and secrete glucagon, while the remaining cells secrete other peptides such as somatostatin and pancreatic polypeptides, whose functions are less well-established. Insulin and glucagon regulate glucose, fatty acid, and amino acid metabolism. Insulin is a peptide hormone consisting of two straight chains linked by two disulfide bridges. The chains are the A chain (with 21 amino acids) and the B chain (with 30 amino acids). A third disulfide bridge, called connecting peptide (C-peptide), is located within the A chain.

Insulin is known as the hormone of abundance or plenty: when the availability of nutrients exceeds the demands of the body, insulin ensures that excess nutrients are stored as glycogen in the liver, fat in adipose tissue, and protein in muscle. Glucagon is the hormone of fasting or starvation: in contrast to insulin; glucagon promotes mobilization and utilization of stored nutrients to maintain blood glucose levels in the fasting state [Costanzo, 2002]. Energy metabolism is controlled primarily by the actions of the hormones insulin, glucagon, epinephrine, and norepinephrine, but the former two hormones are those that most profoundly affect this process [Champe and Harvey, 1987, Costanzo, 2002]. The synthesis and secretion of glucagon increase during fasting, while insulin synthesis and secretion increase during food intake [Champe and Harvey, 1987]. Insulin and cleaved C-peptide are packaged together in secretory granules and, when the beta cells are stimulated, they are released in equimolar

quantities into the blood. C-peptide is thus an indicator of insulin secretion [Kaplan *et al.* 1992].

Vitamin C plays an important role in homeostasis [Kaplan *et al.* 2004a,b,], and studies have demonstrated a relationship between vitamin C and carbohydrate metabolism [Stankova *et al.* 1984, Kaplan *et al.* 1992, Cheng *et al.* 1998]. Like humans, guinea pigs do not synthesize vitamin C [Horning 1981, Kaplan *et al.* 1992]; thus, the guinea pig is an excellent animal model for determining the effects of high-dose vitamin C on carbohydrate metabolism during acute and long-term LFI. The aim of this study was to investigate the glucoregulatory effects of LFI periods and high dose vitamin C supplementation on serum glucose levels, serum C-peptide levels and pancreatic alpha and beta cells of guinea pigs using biochemical and immunohistological methods.

Materials and Methods

Animals

Forty-two guinea pigs weighing 500±25 g were caged in a temperature-controlled room (20-22°C). The animals had free access to water and guinea pig chow during the normal feeding period, and received only a small amount of lettuce (Lactuca sativa; 30 g/day) during periods of LFI. Following the experimental design described below, vitamin C (L-ascorbic acid, Sigma A-7506) supplementation (a single dose of 500 mg/kg body weight by intraperitoneal (i.p.) injection.) [Kaplan 1995] was given just prior to limited food intake (LFI) periods.

In this study, LFI was defined as feeding with 30 g lettuce per day. Lettuce contains mainly fiber and water, and the normal daily consumption for guinea pigs is 60 g/kg. These animals are herbivores and they should be fed ad libitum; they do not adjust well to limit feeding. Guinea pigs require high dietary amounts of various amino acids and these needs are usually met by feeding with a diet of 20% plant-origin protein. In addition, they need to ingest sufficient amounts of fiber (10-18% of the diet) to ensure optimal digestive tract function [Terril *et al.* 1997]. The animals in our study weighed approximately 500 g, so 30 g of lettuce per day was enough to meet their fiber requirements, but not their dietary protein requirements. In addition to amino acids and fiber, the daily diet of guinea pigs must include vitamin C, because they lack one of the enzymes needed to convert glucose to vitamin C

[Kaplan *et al.* 2004a, b]. The longest LFI period (long-term LFI) was 120 h, since the half-life of vitamin C is 96 h in the guinea pig [Horning 1981]. The experimental protocol was approved by the Animal Care Committee and Surgical Research Center of Gazi University Medical School.

Experimental protocol

Seven groups of animals (n = 6/group) were studied. Untreated (control; fed ad libitum); 24 h limited food intake (LFI 24); 48 h limited food intake (LFI 48); 120 h limited food intake (LFI 120); vitamin C supplementation + 24 h limited food intake (Vit C + LFI 24); vitamin C supplementation + 48 h limited food intake (Vit C + LFI 48); vitamin C supplementation + 120 h limited food intake (Vit C + LFI 120). Each guinea pig in the control group was anesthetized with sodium pentothal (0.03mg/kg, i.p.) (Abbott, Italy). Guinea pigs in the other groups were anesthetized by the same procedure after LFI periods. In the control and the other groups, each guinea pig was sacrificed with an overdose of sodium pentothal.

Blood glucose levels

Whole blood samples were obtained by intracardiac puncture. Serum glucose levels were evaluated in an autoanalyzer using the glucose oxidase method [Bauer 1982].

Blood C-peptide levels

Whole blood samples were obtained by intracardiac puncture. There is no commercial kit for measuring insulin in the guinea pig; however, since the primary structure of guinea pig C-peptide is similar to that of other mammals (including humans), C-peptide levels can be measured using a commercial assay [Schlosser *et al.* 1987]. Serum C-peptide levels were determined using a radioimmunoassay (Incstar, lot no: 1500258, for animals), according to the manufacturer's instructions.

Immunohistochemical analysis

After blood was drawn, each animal was killed with an overdose of sodium pentothal; as noted above, the pancreas was removed immediately, and then fixed in 10% buffered formalin. Immunohistochemistry was performed using the streptavidin-biotin immunoperoxidase method. Slides were de-waxed in xylene and hydrated in graded concentrations of alcohol. Endogenous peroxidases were
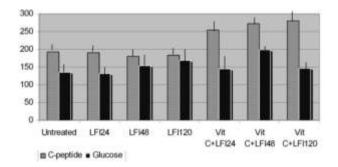
Figure 1. Serum C-peptide levels (pg/L x100) and glucose levels (mg/dL) in guinea pigs after acute (24 h, 48 h) and long-term (120 h) limited food intake (LFI) with or without vitamin C supplementation (Vit C). Data are expressed as the mean ± SE (n= 6). Serum C-peptide levels were significantly lower in untreated (control), LFI 24, LFI 48, LFI 120 than in the Vit C + LFI 24, Vit C + LFI 48 and Vit C + LFI 120 groups (*p*<0.05). The highest serum glucose levels were found in the Vit C + LFI 48 group (*p*<0.05).

Table 1. Percentage and intensity of beta (insulin-secreting) and alpha (glucagon-secreting) cell staining in the islets of Langerhans in guinea pigs after acute (24 h, 48 h) and long-term (120 h) limited food intake (LFI) with or without vitamin C supplementation (Vit C).

Groups	Staining percentage (%)		Staining intensity	
	Beta cells I(+)	Alpha cells G(+)	Beta cells I(+)	Alpha cells G(+)
Untreated (Control)			+	+
LFI 24	70	50-60	+++	+
LFI 48	50-70	30-40	→++	+ →++
LFI 120	20	30-40	+ →++	+++
Vit C + LFI 24	90	10-20	+++	+ →++
Vit C + LFI 48	80	80-90	+ →++	++
Vit C + LFI 120	75	70-90	+ →++	+++

I(+): Beta cells staining (+) for insulin; G(+): Alpha cells staining (+) for glucagon;
+: light staining, ++: moderate staining, +++: intense staining.

blocked with 3% hydrogen peroxide for 10 minutes. After at least 24 h fixation at room temperature, specimens were embedded in paraffin and cut into sections of approximately 4 μm thickness. Hydrogen peroxide was used to block endogenous peroxides. Non-specific antibody binding was blocked by immersing each section in non-immune serum for 5 minutes at room temperature. Primary antibodies to guinea pig insulin (produced in polyclonal guinea pigs; Zymed 08-0067, USA) and to guinea pig glucagon (produced in monoclonal, USA) were provided ready-to-use by the manufacturer. The Histostain broad-spectrum kit (Zymed 95-9942, USA) was used and results were visualized with 3-amino-9-ethylcarbazole (AEC). Counter stain was done with Mayer's hematoxylin. Pancreas was used as positive control for all antibodies. For negative control staining, primary antibody was omitted [Elias 1996].

Statistics

The serum C-peptide and serum glucose data were analyzed using ANOVA and Duncan's multiple range tests. Values were expressed as mean ± SE. *p*<0.05 was considered statistically significant

Results

The serum C-peptide and serum glucose levels results for each group are given in Figure 1. Serum C-peptide levels were significantly lower in untreated, LFI 24, LFI 48, LFI 120 than in the Vit C + LFI 24, Vit C + LFI 48, and Vit C + LFI 120 groups (*p*<0.05). The highest serum glucose levels

were found in the Vit C + LFI 48 group (*p*<0.05). Stained beta cells (insulin-secreting) and alpha cells (glucagon-secreting) were counted in 37 islets of Langerhans from each guinea pig. The intensity analysis for the immunostaining of insulin and glucagon, in the islets (Table 1); light staining (+), moderate staining (++) and intense staining (+++) was done semiquantitatively. Staining intensity was compared with control sections and graded as a light staining, moderate staining and intense staining. The intensity of staining was graded as light (+), moderate (++), or intense (+++) staining was graded as light (+), moderate (++), or intense (+++). The percentage of staining relative to total islet area was also estimated (Table 1).

Immunohistochemical staining intensity of the insulin and glucagon granules between control group were comparable (Figures 2a and 4a). The intensity of insulin granule staining was lower in the LFI 24 animals than in the Vit C + LFI 24 animals (Figures 2b and 3a). However, the LFI 24 group showed more intense staining for glucagon than the Vit C + LFI 24 group (Figures 4b and 5a). The staining intensity of insulin granules in the LFI 48 samples was lower than that observed in the Vit C + LFI 48 animals (Figures 2c and 3b). There was also less intense staining of glucagon granules in the LFI 48 group than in the Vit C + LFI 48 group (Figures 4c and 5b). Similarly, in the long-term LFI animals, staining intensity for both insulin and glucagon was lower in the LFI 120 group than in the Vit C + LFI 120 animals (respectively, Figures 2d and 3c for insulin and 4d and 5c for glucagon).

Discussion

Many studies have examined the influence of vitamin C on carbohydrate metabolism. Abdel-Wahab et al. [2002] showed that vitamin C supplementation decreased insulin glycation and improved glucose homeostasis in obese hyperglycemic mice. It has also been shown that intravenous injection of vitamin C (100 mg/kg) markedly reduces the blood glucose concentration in anesthetized rats and that this hypoglycemic effect is accompanied by increased serum insulin concentrations [Cheng et al. 1988]. Other studies have revealed that intra-venous or oral administration of vitamin C produces a hypoglycemic effect in diabetic patients [Burns et al. 1995] and in guinea pigs [Chattarjee et al. 1975]. In contrast, there is relatively little information about the effect of vitamin C on pancreatic function.

In this study, we investigated the relationship between vitamin C supplementation and pancreatic tissue, using biochemical, histological, and immuno-histochemical examination of the pancreatic islets of guinea pigs on LFI, with or without vitamin C supplementation, for various time periods. LFI
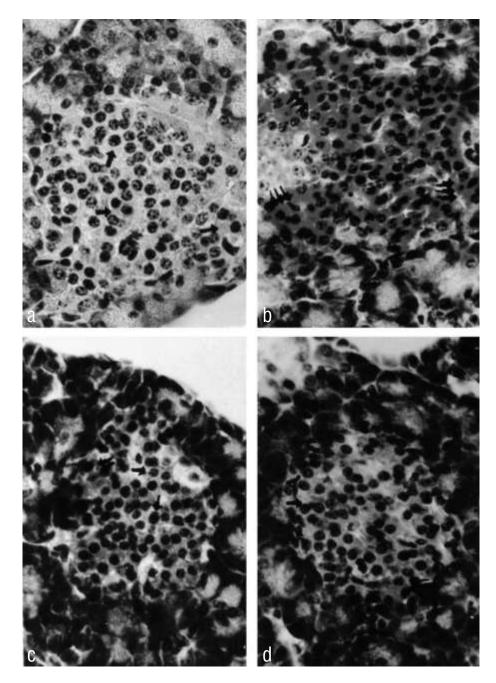

Figure 2. Immunohistochemical staining of insulin shows beta cells in the islets of Langerhans of a pancreas from an untreated guinea pig (a, single arrow), an LFI 24 animal (b, triple arrows), from an LFI 48 animal (c, single and double arrows), and from an LFI 120 animal (d, single and double arrows). Staining is alkaline phosphatase red; magnification is 400x).
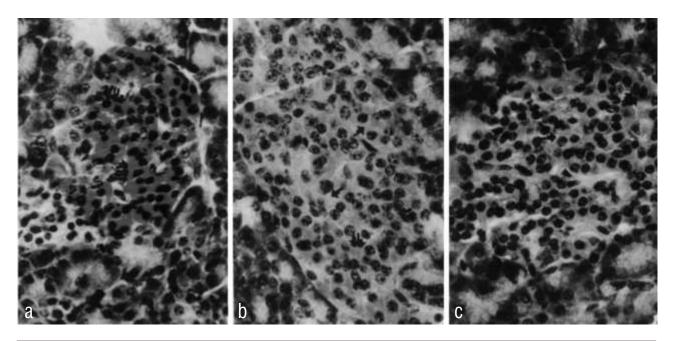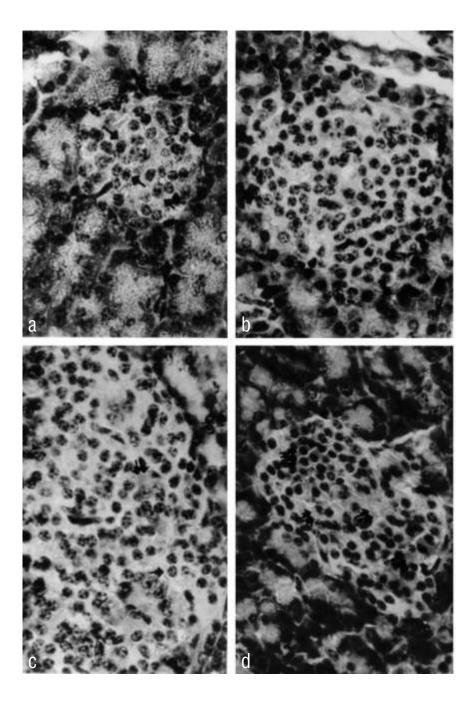
Figure 3. Immunohistochemical staining of insulin shows beta cells in the islets of Langerhans of a pancreas from a Vit C + LFI 24 guinea pig (a, triple arrows), a Vit C + LFI 48 animal (b, single and double arrows), and from a Vit C + LFI 120 animal (c, single and double arrows). Staining is alkaline phosphatase red; magnification is 400x).
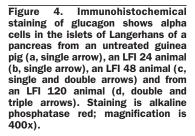
induces stress. During exposure to stressful conditions, the sympathetic nervous system is activated, with a response known as *fight or flight* [Costanzo 2002]. During stress, adrenergic neurons of the autonomic nervous system release norepinephrine (NE), the receptors for which (called adrenoceptors) are located in the effector organs. Adrenoceptors may be activated by NE or by epinephrine, which is secreted into the circulation by the adrenal gland. The adrenal medulla secretes mainly epinephrine (80%) and a much smaller amount of NE (20%) [Vander *et al.* 2001, Costanzo 2002]. Alan [1993] reported that dopamine-β-hydroxylase, which catalyzes the final step in the biosynthesis of NE, uses vitamin C as the electron donor in reducing molecular oxygen to hydroxyl oxygen. This is an important step in NE production.

We administered vitamin C intraperitoneally during acute and long-term LFI. We found that the immunopositive beta cells made up 70% of the islets of Langerhans in the LFI 24 group. After treatment with vitamin C, this figure rose to 90%. This increase indicates that vitamin C boosts beta cell activity, stimulating increased synthesis and storage of insulin.

It is known that insulin inhibits glucagon secretion [Costanzo 2002]. In addition, the staining percentage for alpha cells was 50-60% in the LFI 24

animals, a figure which dropped to 10-20% after vitamin C supplementation, suggesting that the elevation of insulin levels decreased the synthesis and secretion of glucagon in the Vit C + LFI 24 group. C-peptide is produced in concentrations eqimolar to insulin, and serum C-peptide levels are an index of insulin secretion [Schlosser *et al.* 1987, Ganong 2003]. We have demonstrated that the elevation of serum C-peptide levels decreased glucagon levels in the Vit C + LFI 24 animals. Our data indicate that insulin synthesis, storage, and secretion are increased by vitamin C supplementation and the stress induced by LFI 24.

Our data indicate that NE synthesis may have been increased by vitamin C supplementation and the stress induced by LFI 24 . Stimulatory effects on glucagon secretion are mediated in part by activation of sympathetic alpha-adrenergic receptors [Champe and Harvey 1982, Costanzo 2002]. The staining intensity for alpha cells in the LFI 48 animals was noticeably lower than that in the Vit C + LFI 48 animals. Thus, glucagon secretion was increased by vitamin C supplementation in the LFI 48 animals. We showed that alpha cell staining in the Vit C + LFI 24 animals was less intense than that in the Vit C + LFI 48 animals, indicating that glucagon secretion was lower in the former group. We found the highest serum glucose levels in the Vit C + LFI 48 group. It is known that glucagon helps

Figure 4. Immunohistochemical staining of glucagon shows alpha cells in the islets of Langerhans of a pancreas from an untreated guinea pig (a, single arrow), an LFI 24 animal (b, single arrow), an LFI 48 animal (c, single and double arrows) and from an LFI 120 animal (d, double and triple arrows). Staining is alkaline phosphatase red; magnification is 400x).

maintain blood glucose levels by activating hepatic glycogenolysis and glyconeogenesis during fasting, and perhaps during LFI [Champe and Harvey 1982, Costanzo 2002]. Our data indicate that the elevation of glucagon increased blood glucose. Glucagon stimulates insulin secretion [Costanzo 2002]. We showed that insulin synthesis, storage, and secretion were stimulated in the pancreatic beta cells of Vit C + LFI 48 guinea pigs. In addition, we found that the Vit C + LFI 24 animals had significantly lower serum C-peptide levels than the

Vit C + LFI 48 animals. Thus, it is possible that the C-peptide secretion from beta cells are increased by vitamin C and glucagon, especially when vitamin C is supplemented before a 48 h period of LFI. We know that insulin inhibits glucagon secretion [Costanzo 2002]. The secretion of glucagon by alpha cells is markedly decreased by elevated blood sugar, while insulin limits the rise in blood glucose [Champe and Harvey 1982, Costanzo 2002]. Our data suggest that the elevation of glucagon secretion in the Vit C + LFI 48 animals may have been
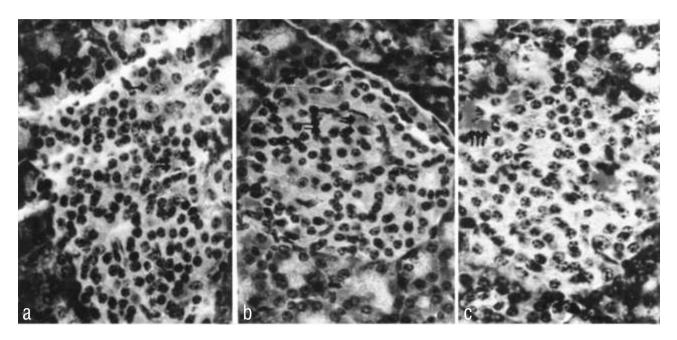
Figure 5. Immunohistochemical staining of glucagon shows alpha cells in the islets of Langerhans of a pancreas of a Vit C + LFI 24 guinea pig (a, single and double arrows), a Vit C + LFI 48 animal (b, double arrow) and from a Vit C + LFI 120 animal (c, triple arrow). Staining is alkaline phosphatase red; magnification is 400x).

limited by the serum glucose level by increasing insulin levels in this group. These findings suggest that LFI-induced stress and high-dose vitamin C may have increased NE biosynthesis, thus increasing glucagon synthesis, storage, and secretion, which in turn increased blood glucose and insulin synthesis, storage, and secretion.

In the Vit C + LFI 120 group, the staining for both insulin and glucagon granules was significantly more intense than that observed in all the other LFI groups. The lifespan of vitamin C in guinea pigs is 2.5–6.0 days [Englard and Seifter 1986]. We found that alpha cells showed more intense staining for glucagon than beta cells for insulin depended on Vit C + LFI 120. This suggests that glucagon synthesis, storage and secretion increased in order to maintain glucose homeostasis at the end of the vitamin C life span or in the Vit C + LFI 120 group.

In conclusion, we suggest that high doses of vitamin C and LFI-induced stress may increase NE biosynthesis, which can increase glucagon secretion, depending on the period of LFI (especially vitC+ LFI 48), which increases blood glucose, which in turn elevates C-peptide secretion. Thus, NE elevation may increase glucagon synthesis and secretion; in turn, higher levels of circulating glucagon will cause an elevation of blood glucose and cause C-peptide secretion (an index of insulin secretion) to increase. Large doses of vitamin C in conjunction with LFI may, therefore, indirectly affect blood glucose regulation in the maintenance of homeostasis.

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