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Research Article

Effects of Anesthesia on Hyperglycemic and Insulinotropic Responses to Intravenous Glucose and Mixed Meal Tolerance Tests in Cynomolgus Monkeys with Naturally Occurring Diabetes -

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ABSTRACT

Intravenous Glucose (ivGTT) and Mixed Meal (MMTT) tolerance tests are commonly used to evaluate glucose homeostasis in both human and laboratory animals. Similar to that in type 2 diabetic (T2D) patients, we used these tests to have demonstrated abnormal glucose control in Non-Human Primate (NHP) model of spontaneously developed diabetes, a translatable model commonly used for assessing anti-diabetic therapeutics. However, the effects of anesthesia on glucose or hormonal responses during these tests are not known. The aim of the present study was to examine the impact of anesthesia on metabolic and hormonal responses to ivGTT (n = 5) and MMTT (n = 10) in male cynomolgus monkeys with naturally occurring diabetes and their normoglycemic controls in the presence or absence of ketamine (5mg/ kg) anesthesia when glucose, insulin, entero-gastric hormones, adipokines and inflammatory cytokines were measured. Diabetic NHPs developed impaired glucose tolerance in both ivGTT and MMTT. During ivGTT, anesthesia enhanced insulin response accompanied by an increase in glucose clearance rate only in the control but not diabetic NHPs. In contrast, during MMTT, anesthesia appeared to reduce the glucose response in the control NHPs accompanied by reductions in serum levels in GIP, leptin, resistin, visfatin, IL-6 and MCP-1. These data demonstrated that both ivGTT and MMTT can serve as a powerful tool to accurately examine glucose homeostasis in the progression of diabetes in the NHP model. Anesthesia may impact the degree of these tests differently in diabetic and normoglycemic NHPs.

Keywords: Monkeys; Diabetes; Insulin; Glucose; Glucose tolerance test; Mixed meal tolerance test; Gut hormones

ABBREVIATIONS

AAALAC: Assessment and Accreditation of Laboratory Animal Care; AUC: Area under the Curve; GLP-1: Glucagon like Peptide-1; GGI: Graded Glucose Infusion; GIP: Gastric Inhibitory Polypeptide; HbA1c: Hemoglobin A1c; IACUC: Institutional Animal Care and Use Committee; IL-6: Interleukin 6; ivGTT: intravenous Glucose Tolerance Test; LPL: Lipoprotein Lipase; MCP-1: Monocyte Chemotactic Protein-1; MMTT: Mixed Meal Tolerance Test; NASH: Non-Alcoholic Steatohepatitis; NHP: Non-Human Primate; oGTT: oral Glucose Tolerance Test; SE: Standard Error; TNF- α : Tumor Necrosis Factor- α ; T2D: Type 2 Diabetic

INTRODUCTION

Elevation of blood glucose concentration by means of glucose intake stimulates pancreatic secretion of insulin and inhibits its glucagon release, which is regulated by entero-insular axis, a complex hormonal response [1,2]. Intravenous (i.v.) administration of glucose, such as i.v. Glucose Tolerance Test (ivGTT) [3,4], graded glucose infusion test (GGI)[5-7], hyperglycemic clamp[8,9], etc. are direct ways to elevate blood glucose concentration to induce glucose-stimulated insulin release from pancreatic β cells. However, these approaches to stimulate insulin release may not effectively involve entero-insular axis, since many of the incretin hormones such as gastric inhibitory peptide (GIP), glucagon like peptide 1 (GLP-1), ghrelin, etc. are produced from the enteric cells of the gut and secreted into the blood circulation with the ingestion of glucose and other nutrients, such as proteins and lipids [1, 10-12]. Circulating GLP-1 and GIP exert their glucose lowering effects directly by binding to their respective receptors in target tissues, such as pancreatic β and α cells to regulate insulin and glucagon release [10,13,14] or indirectly by modulating gastric emptying hence slowing down the absorption of nutrients [15]. Therefore, oral glucose (oGTT) or mixed meal (MMTT) tolerance test gives a more comprehensive assessment not only for the functions of β or α cells, but also for the impact of entero-insular axis, such as GLP-1 and other incretin signalling [8,16,17]. MMTT is commonly used to assess β cell functions in diabetic patients [18,19], and animal models [20,21]. Compared to oral glucose tolerance test (oGTT), the mixed meal adopted in the MMTT contains multiple nutrients, such as proteins and lipids in addition to glucose that are involved in metabolic homeostasis, thus resembling the post-prandial energy intake in a more physiologically relevant manner [19].

For laboratory experiments using animal models, especially Nonhuman Primates (NHPs), many studies, such as GTTs, GGI, hyperglycemic clamps, etc. often applied anesthesia to avoid impact of multiple procedure-induced stress on the experimental outcomes [22-25]. Thus, the effects of disease progress and therapeutic treatments on blood glucose concentration can sometimes be masked by procedure-induced stress, which can otherwise be improved by application of proper anesthetics [23,25]. Ketamine is a commonly used injectable anesthetics in animal researches with the advantage of low incidences of overdosing and fast onset of sedation [22,24,26,27]. In addition to its anesthetic and analgesic effects, ketamine has been suggested to affect lipid (28)and glucose metabolism possibly by regulating adipokines [29-32] and cytokines[33]. Moreover, ketamine induced anesthesia slows down gastric emptying, hence indirectly affecting release of entero-gastric hormones such as GLP-1 and GIP, which are important regulators in controlling glucose metabolism [34,35]. Thus procedures that use anesthesia for evaluation of glucose metabolism in lab NHPs should be carefully assessed for their impact on glucose homeostasis.

The spontaneously diabetic cynomolgus monkey (*Macaca Fascicularis*) has been recognized as a highly translatable pre-clinical model for evaluations of potential therapeutics in the treatment of metabolic diseases such as diabetes [9,36], obesity[37], hyperlipidemia and non-alcoholic steatohepatitis (NASH)(manuscripts under review). These monkeys displayed changes in metabolic parameters such as blood glucose, HbA1c, insulin, c-peptide, lipid levels and urine indexes in patterns and responses to standard therapy that mimic human patients [7,9,36,37]. Our lab has previously shown that in conscious animals, diabetic cynomolgus monkeys are glucose intolerant with diminished insulin responses to ivGTT [9]. Their responses to MMTT involving gut interaction with nutrients other than pure glucose were not known. Moreover, what factors might contribute to the possible differences in their respective responses and if anesthesia might render different metabolic impacts during ivGTT and MMTT were not understood.

Therefore, the present study aimed to investigate the impact of anesthesia on metabolic responses during both ivGTT and MMTT in normoglycemic and diabetic cynomolgus macaques. These results will help guide the choice of proper glucose or nutrient tolerance test and the decision of using anesthesia for best evaluating the post-prandial glucose response in the diabetic NHPs.

MATERIALS AND METHODS

Animals

Thirty male cynomolgus macaques (*Macaca fascicularis*) without history of pharmaceutical treatment in the past 3 months, and with stable physiological conditions were selectively used in the study. Only male animals were selected in the study to avoid impact from female menstrual cycles. These animals were individually housed at room temperature 21-23°C and 7:00 – 19:00 light and dark cycle with free access to water ad libitum twice daily nutritionally balanced monkey diet (Shanghai Shilin Biotechnology, Inc., Shanghai, China) enriched with seasonal fruits and vegetables. The experimental protocol for the use of monkeys was in accordance with the guidelines of Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) and approved by the local Institutional Animal Care and Use Committee (IACUC).

General characteristics of experimental animals

As humans and other great apes (*Hominoidea*) diverged from *Cercopithecoidea* [38], obesity, dysmetabolism and diabetes occur in Old World monkeys, which would thus provide a good research model to study human diabetes [39,40]. NHPs raised in indoor cages increase the chance to become diabetic and facilitate their development to diabetes-associated diseases in an age-dependent manner when given food ad libitum [39,40]. Like humans, these NHPs develop Type 2 diabetes mellitus (T2D) and other complications, such as nephropathy, ophthalmopathy, neuropathy, cardiovascular complications, etc. [9,36,37,41]. Based on previous characterization [9], normoglycemic (control) and diabetic cynomolgus macaques (diabetic) were selected for the study and their age, body weight, Body Mass Index (BMI), waist circumference, HbA1c and fasting blood glucose levels were listed in (Table 1).

Intravenous glucose tolerance test (ivGTT)

Animals were trained daily by sitting in the restraining chairs for 2 hours for one week before the test. Animals were fasted overnight (~16 h) before the test. On the days of ivGTT, animals were either restrained to monkey chairs during the entire test (30 min) or anesthetized with intramuscular administration of 10-15 mg/kg ketamine. Dextrose (50%, 0.25g/kg) was injected intravenously. Blood from cephalic vein was collected following time points at 0 (pre-dosing), 3, 5, 7, 10, 15, 20 and 30 min post-dosing for blood glucose and insulin measurement. The animals behaved calm during the repetitive bleeding in the test. The animals were then returned to their cages and were monitored until recovering from anesthesia.

Mixed meal tolerance test (MMTT)

Animals were trained daily by sitting in the restraining chairs for 2 hours for one week before the test. Animals were fasted overnight (~16 h) before the test. On the days of MMTT, animals were either restrained to monkey chairs during the entire test (180 min) or were anesthetized with intramuscular administration of 10-15 mg/kg ketamine. After animals were secured on the monkey chairs or anesthetized, a mixed meal consisting of Ensure Plus (350 calories, Abbott, US) with additional sucrose (24 g per 250 ml) was administered via a nasogastric tube with a volume of 5 ml/kg. Blood from cephalic vein was collected following time points at 0 (pre-dosing), 10, 20, 30, 60, 90, 120 and 180 min post-dosing for blood glucose and other serum analysis. The animals behaved calm during the repetitive bleeding in the test. The animals were then returned to their cages and were monitored until recovering from anesthesia.

Serum analysis

Blood collected from the cephalic vein during the study was centrifuged at 3000 rpm for 10 min at 4 °C and serum extracted. Serum GLP-1, GIP, glucagon, ghrelin, leptin, resistin, visfatin, IL-6 and MCP-1 were measured by multiplex technology (Luminex 200) using Bio-Plex Pro Human Diabetes 10-Plex Assay, Human Metabolic Panel 1, Human Chemokine Panel (Bio-Rad, Shanghai, China). Insulin levels were analyzed by a human insulin ELISA kit (ALPCO, US).

Statistical analysis

All data were expressed as mean ± Standard Error (SE). Statistical analysis was performed using unpaired student t test or one-way or two-way ANOVA with post hoc comparison. P-values less than 0.05 were considered statistically significant.

RESULTS

General characteristics (Table 1)

The fasting blood glucose was significantly higher in the diabetic than control NHPs with comparable body weight. In the MMTT experiment, the diabetic NHPs were older with greater BMI and waist circumferences compared to those of the controls. In general, diabetic animals were older than the controls.

Intravenous glucose tolerance test (Figure 1)

Glucose intolerance in the diabetic NHPs

The baseline glucose levels were not significantly affected by anesthesia in either the control (Figure 1A) or diabetic (Figure 1B) groups. Upon i.v. glucose challenge, blood glucose increased sharply followed by a gradual decline to the baseline after 30 min only in the control (Figure 1A), but not diabetic (Figure 1B) group. A significantly greater area under the time course of glucose concentration curve (AUC) revealed glucose intolerance in the diabetic NHPs, which was not significantly affected by anesthesia in both groups (Figure 1C).

Anesthesia enhanced insulinotropic response and glucose clearance in the control NHPs

The basal insulin levels were similar between the control (Figure 1E) and diabetic (Figure 1F) NHPs, which were not affected by

Table 1: General characteristics of the control and diabetic monkeys for intravenous glucose (ivGTT) and oral mixed meal (MMTT) tolerance tests.

ivGTT	Control (n=5)	Diabetes (n=5)	P values
Age (years)	11.5 ± 2.4	17.3 ± 2.2	0.21
Body weight (kg)	7.5 ± 1.1	8.3 ± 1.3	0.35
HbA1c (%)	4.6 ± 0.1	7.1 ± 0.9	0.03
Fasting blood glucose (mg/dl)	68.4 ± 3.6	205.2 ± 30.6	0.00
MMTT	Control (n=10)	Diabetes (n=10)	P values
Age (years)	8.8 ± 1.1	17.8 ± 0.8	0.00
Body Weight (kg)	7.4 ± 0.5	7.6 ± 0.6	0.87
Body Mass Index (kg/m ²)	14 ± 0.8	18 ± 0.9	0.01
Waist Circumference (cm)	40 ± 1.7	48 ± 1.6	0.01
HbA1c (%)	4.6 ± 0.1	6.5 ± 0.6	0.00
Fasting blood glucose (mg/dl)	63.6 ± 3.05	114.3 ± 8.7	0.00

P value was calculated between control and diabetic monkeys using unpaired student t test.

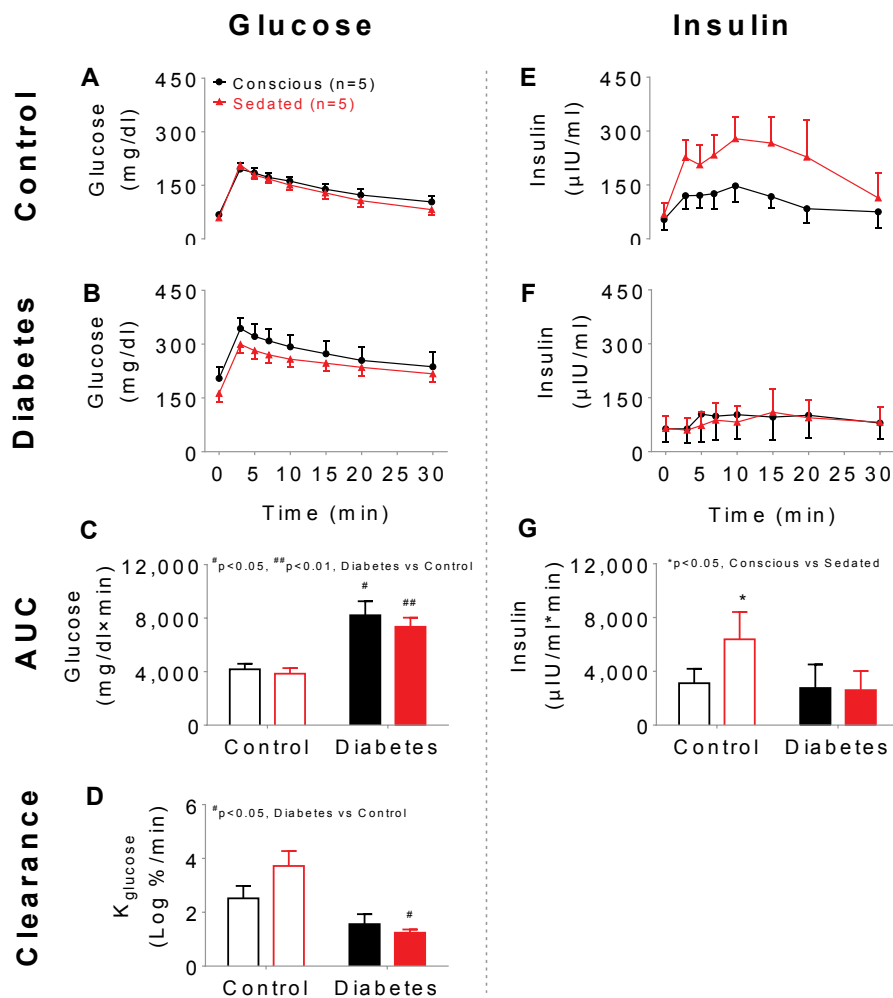


Figure 1: Effects of anesthesia on glucose and insulin response to Intravenous Glucose Tolerance Test (ivGTT) in the control and diabetic cynomolgus monkeys. Blood glucose (A & B) and serum insulin (E & F) response curves, their respective area under the curve (AUC) (C & G) and glucose disappearance rate (D) following intravenous glucose challenge (0.25 g/kg glucose).

anesthesia. Despite a much greater hyperglycemic response (Figure 1B), the insulin response appeared blunted in the diabetic NHPs (Figure 1F). However, anesthesia significantly enhanced insulin response, hence increased glucose clearance rate (Figure 1D) only in the control (Figure 1E), but not in the diabetic (Figure 1F) group (Figure 1G).

Mixed meal tolerance test (MMTT)

Anesthesia suppressed hyperglycemic responses in the control NHPs (Figure 2)

Oral gavage of NHPs with liquid formulation containing mixed ingredients led to a slow increase in blood glucose followed by a gradual decline in both the control (Figure 2A) and diabetic (Figure 2B) NHPs with the response significantly greater in the diabetic group (Figure 2C). The anesthesia markedly suppressed MMTT-induced hyperglycemic response with a significantly reduced AUC only in the control, but not diabetic NHPs (Figure 2C). There were no significant differences in baseline and insulin response between the two groups (Figure 2D-F). Anesthesia only slightly reduced insulin response in

the control (Figure 2D), but not diabetic (Figure 2E) NHPs (Figure 2F).

Anesthesia suppressed some incretin, cytokine and adipokine responses

The basal levels of the gut hormones and cytokines measured were comparable between the control and diabetic NHPs (Figure 3). MMTT markedly increased the serum concentrations of GIP (Figure 3A&B) but not GLP-1 (Figure 4A&B) and ghrelin (Figure 4D&E) in both the control and diabetic NHPs. Anesthesia, however, significantly suppressed the responses for GIP in both the control (Figure 3A) and diabetic (Figure 3B) monkeys (Figure 3C), but not GLP-1 (Figure 4A-C) or Ghrelin (Figure 4D-F).

MMTT led to a 3-fold increase in IL-6 (Figure 3D) and MCP-1 (Figure 3G) levels 180 min after the meal in the conscious control NHPs, while these responses in diabetic ones were relatively mild (Figure 3E& H). Anesthesia dramatically suppressed the responses of IL-6 (Figure 3D) and MCP-1 (Figure 3G) in the controls, while its effect in the diabetic animals (Figure 3E&H) was much less pronounced (Figure 3F & I).

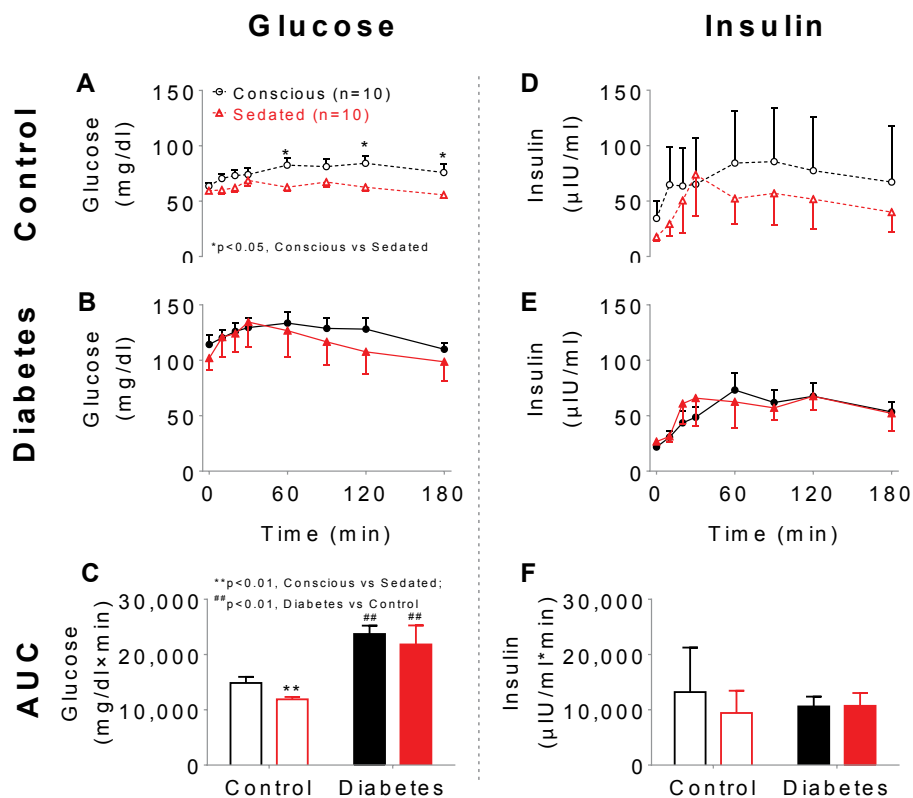


Figure 2: Effects of anesthesia on glucose and insulin response to oral Mixed Meal Tolerance Test (MMTT) in the control and diabetic cynomolgus monkeys. Blood glucose (A & B) and serum insulin (D & E) response curves and their respective area under the curve (AUC) (C & F) following an oral mixed meal challenge.

The serum concentrations of leptin (Figure 5A& B), resistin (Figure 5D& E) and visfatin (Figure 5G&H) were comparable at baseline between the control and diabetic groups, which were slowly increased at 180 min during MMTT in the controls with a much mild response in diabetics. Anesthesia suppressed MMTT responses for all the three adipokines in the control (Figure 5A, D, G), but not diabetic (Figure 5B, E, H) NHPs (Figure 5C, F, I).

DISCUSSION

Glucose intolerance during both ivGTT and MMTT in the diabetic NHPs

The present as well as our previous studies demonstrated that diabetic NHPs developed poorer ability in controlling glucose homeostasis in response to ivGTT. The present data further established that MMTT which involves gastro-enteric hormones can also well distinguish the diabetic NHPs with enhanced hyperglycemic response. In consistent with T2D patients, ingestion of a mixed meal containing 445kcal energy resulted in a doubled increase in blood glucose comparing to normal individuals [20,42]. Unlike ivGTT where the insulin response diminished in the diabetic NHPs, its response to MMTT were comparable between the conscious control and diabetic animals.

Anesthesia restored insulinotropic response to ivGTT in the control NHPs

Unlike in human whose stress responses are normally low during

ivGTT[43,44], restraining procedures in conscious NHPs can induce excessive stress which may lead to undesired elevation in blood glucose levels. One easy approach to the problem is to run the study under anesthesia (23; 45). Though there are reports suggesting that anesthesia can reduce stress-induced glucose hike and hormonal changes during ivGTT in NHPs [22-24,26,27,45], the present data failed to show marked effects of anesthesia on the hyperglycemic response during ivGTT in either the control or diabetic NHPs, however, the glucose-stimulated insulin response was significantly enhanced by anesthesia in the control, but not diabetic group. This could be due to the fact that the restraining procedure-induced stress in the conscious NHPs suppressed the glucose-stimulated insulin response, while anesthesia relieved such stress, thus restored the insulin response to normal/higher level, which in turn further restored/enhanced the glucose clearance rate in the control NHPs. In the diabetic NHPs, the pancreatic function was already compromised, which cannot be further enhanced.

Anesthesia suppressed the hyperglycemic response to MMTT in the control NHPs

Unlike ivGTT, MMTT involves the gut absorption of the glucose and nutrients. It is well known that anesthesia could reduce gastric emptying hence slow down the absorption of glucose and other nutrients into the blood [46-48]. Indeed, the present data demonstrated that anesthesia reduced both hyperglycemic and insulinotropic (though in a less degree) responses to MMTT in the control, but not in diabetic NHPs. Since the diabetic NHPs already

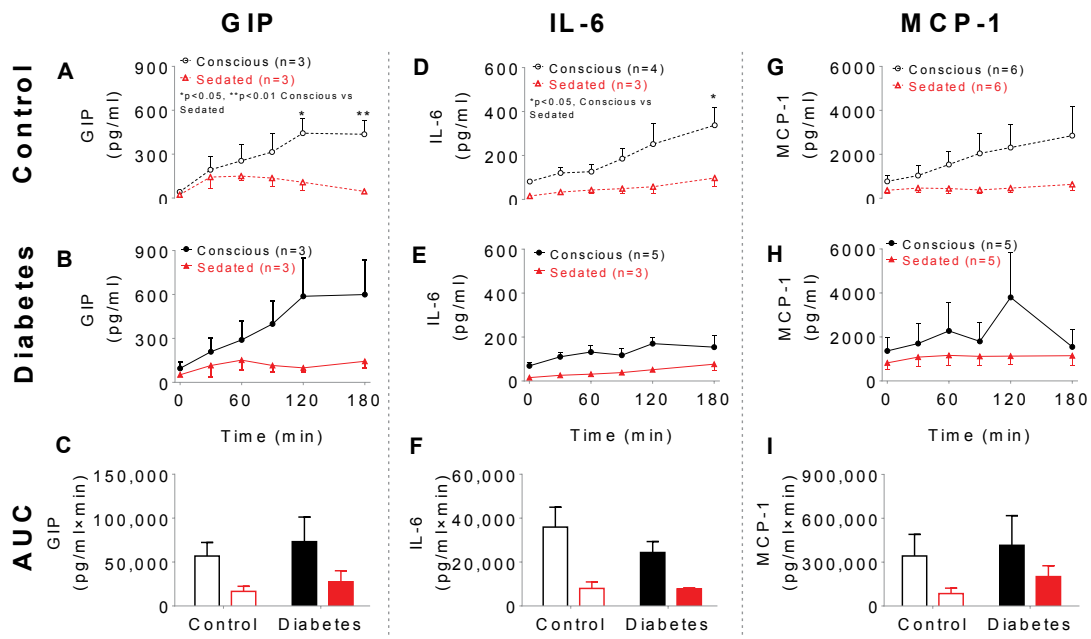


Figure 3: Effects of anesthesia on GIP, IL-6 and MCP-1 responses to oral Mixed Meal Tolerance Test (MMTT) in the control and diabetic cynomolgus monkeys. Serum GIP (A & B), IL-6 (D & E) and MCP-1 (G & H) response curves and their respective area under the curve (AUC) (C, F & I) following an oral mixed meal challenge.

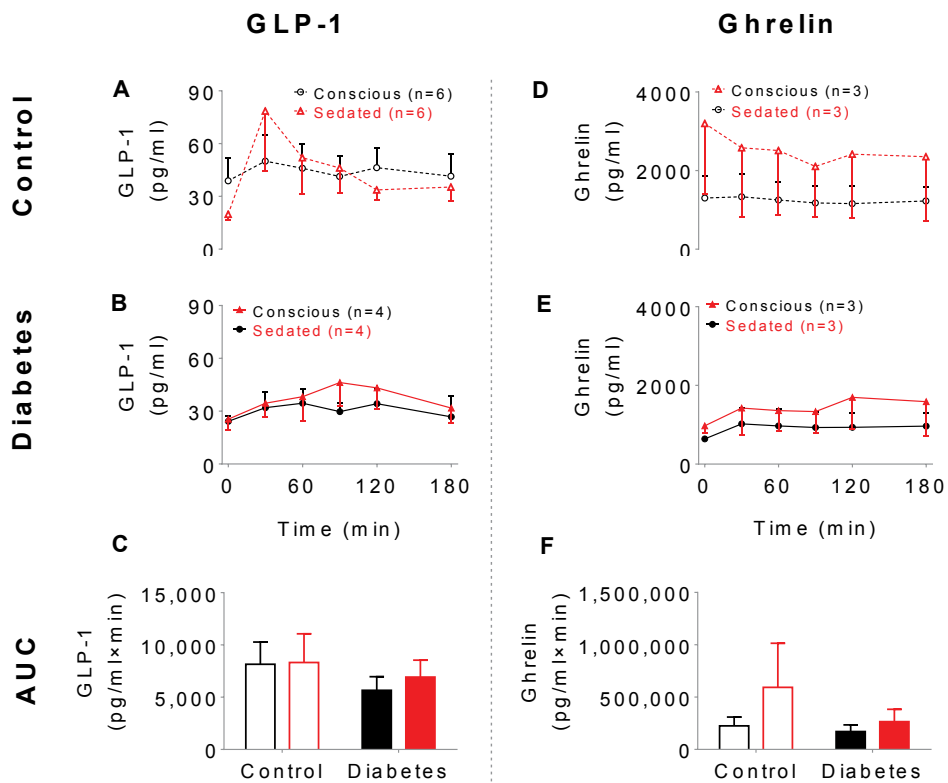


Figure 4: Effects of anesthesia on GLP-1 and ghrelin responses to oral Mixed Meal Tolerance Test (MMTT) in the control and diabetic cynomolgus monkeys. Serum GLP-1 (A & B) and ghrelin (D & E) response curves and their respective area under the curve (AUC) (C & F) following an oral mixed meal challenge.

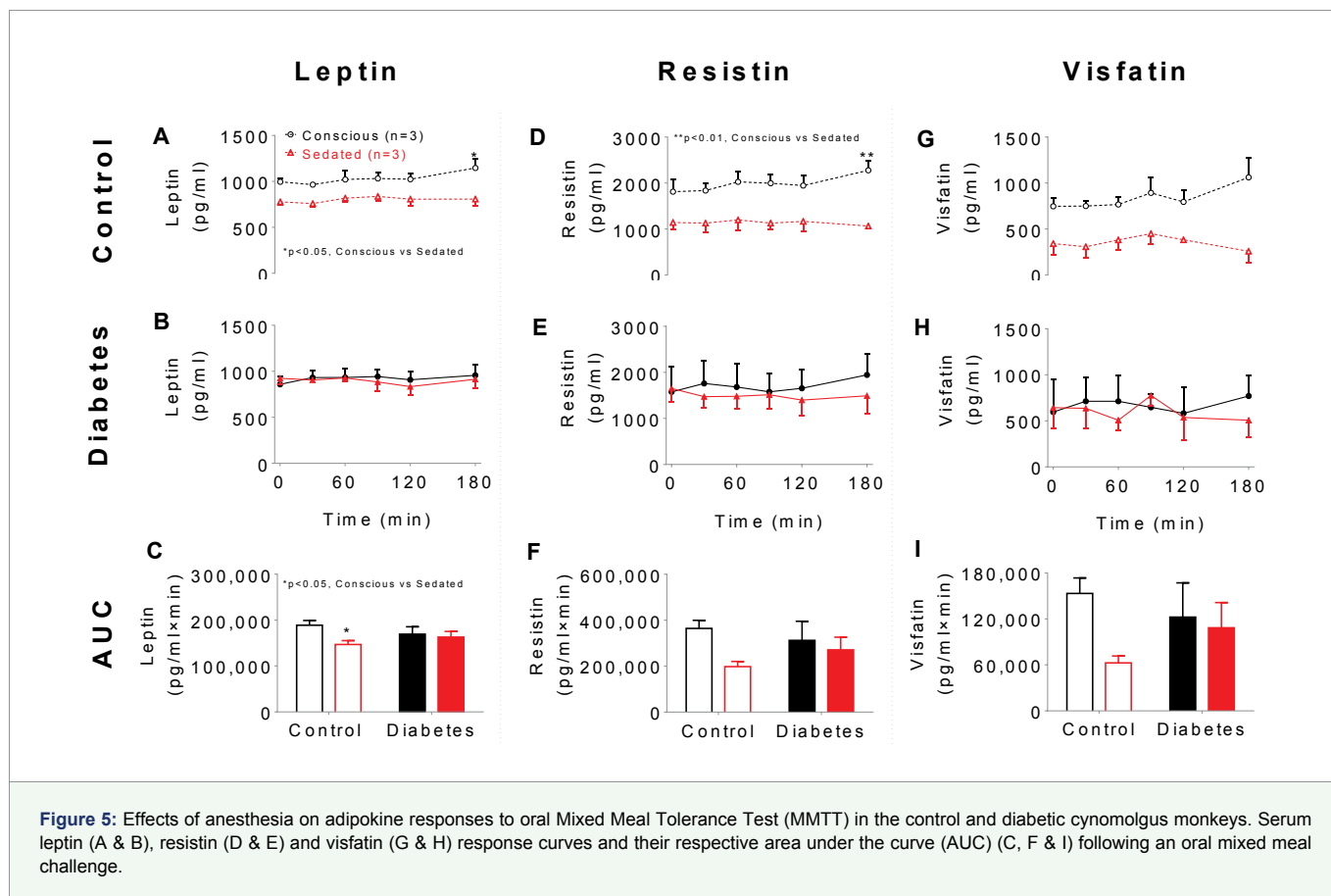


Figure 5: Effects of anesthesia on adipokine responses to oral Mixed Meal Tolerance Test (MMTT) in the control and diabetic cynomolgus monkeys. Serum leptin (A & B), resistin (D & E) and visfatin (G & H) response curves and their respective area under the curve (AUC) (C, F & I) following an oral mixed meal challenge.

had severely impaired mixed meal tolerance as well as compromised pancreas function, the responses cannot be further reduced by a slow-down in the absorption of glucose and nutrients during anesthesia.

Role of gut hormones, adipokines and cytokines in regulation of glucose homeostasis

MMTT also involves the interaction of glucose and nutrients with the gut to stimulate the release of a series of glucose- and satiety-controlling hormones from the gastric and enteric cells, including GIP, GLP-1, ghrelin etc. [12,17,49]. These entero-gastric hormones, along with adipokines and cytokines induced during meal intake can regulate glucose metabolism, which could also be affected by anesthesia [29-31,34,46,51-53]. The present data indeed demonstrated that GIP, leptin, resistin, visfatin, IL-6 and MCP-1, but not GLP-1 and ghrelin were increased during MMTT, which were dramatically suppressed by anesthesia in the control NHPs. This was in sharp contrast to the diabetic animals whose levels of adipokines and cytokines were readily suppressed, thus, cannot be further reduced by anesthesia.

Incretin The levels of GIP, GLP-1 and ghrelin in the present study were similar between the control and diabetic groups. This result is somewhat different from what has been reported in T2D patients where GIP changes were lower in the diabetic than healthy controls during MMTT [20]. However, the stress response in the conscious NHPs and different composition of the ingredients used in the mixed meal might contribute to such discrepancy. It is intriguing to see the responses of GIP, but not GLP-1 and ghrelin to MMTT were significantly attenuated by anesthesia, as reducing gastric emptying ought to reduce both GIP and GLP-1, hence lowering glucagon

levels [10,13,14,34], suggesting that inhibition of GIP secretion by anesthesia may be independent of gastric emptying mechanism, though the exact mechanism is still not known.

Cytokines The present data also showed that MMTT-induced increases in IL-6 and MCP-1 were not as pronounced in the diabetic comparing to control NHPs. The changes of post prandial IL-6 in T2D patients were somewhat inconsistent in different studies as its levels decreased in Indian healthy and T2D patients [50], or elevated in glucose-impaired patients [51]. Such discrepancy might be reflected by the differences in meal compositions, treatment background, individual heterogeneity of the patients recruited. In contrast, the genetic background of the NHPs for the experimental models is more homogenous. MCP-1 has been found significantly increased in T2D patients [52]. An A/G polymorphism of MCP-1 which affects its expression correlated well with the reduction of prevalence of insulin resistance and T2D, suggesting a role of MCP-1 in regulating glucose metabolism [53,54]. Though the exact causes of the discrepancy of IL-6 changes between NHPs and human after a meal are not well defined, reduced IL-6 and MCP-1 might partially contribute to the mixed meal intolerance in the diabetic NHPs.

Adipokines Ketamine is a commonly used anesthetic in laboratory animals [24]. It can deposit in white adipose tissues while stimulating lipolysis as evidenced in swine adipose tissues [28]. Further studies performed in rats suggested that high doses of ketamine can reduce lipoprotein lipase activities [55]. The secretion of adipokines such as leptin, resistin and visfatin from adipose tissues can, therefore, be affected by ketamine to regulate lipid metabolism of adipose tissues. The changes of these adipokines in the control NHPs

during anesthesia implied that the control animals may be more sensitive to anesthesia than the diabetic ones in regulating adipokine secretions.

CONCLUSION

Spontaneously developed diabetic cynomolgus macaques showed impaired glucose tolerance in ivGTT and MMTT. Anesthesia may relieve stress-induced suppression of the insulinotropic response to ivGTT, however, it may also reduce the absorption of the glucose and nutrients via inhibiting gastric emptying, hence reducing the secretion of incretin hormones, as well as adipokines and cytokines that involved in glucose metabolism. Thus, naturally occurring diabetic cynomolgus macaques coupling with the tools such as ivGTT and MMTT and the use of anesthesia can be an excellent metabolic disease model for pre-clinical anti-diabetic drug researches.

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