Introduction
Diabetes, characterized by elevated glucose secondary to a combination of insulin resistance and decreased insulin secretion, is a serious worldwide health problem [1]. This disease has an excess impact on low- and middle-income countries and is associated with a significant increase in cardiovascular and microvascular complications and premature mortality [1].

ABSTRACT
Diabetes is a life-threatening and debilitating disease with pathological hallmarks, including glucose intolerance and insulin resistance. Plant compounds are a source of novel and effective therapeutics, and the flavonoid (-)-epicatechin, common to popular foods worldwide, has been shown to improve carbohydrate metabolism in both clinical studies and preclinical models. We hypothesized that (-)-epicatechin would alleviate thermoneutral housing-induced glucose intolerance. Male rats were housed at either thermoneutral (30 °C) or room temperature (24 °C) for 16 weeks and gavaged with either 1 mg/kg body weight or vehicle for the last 15 days before sacrifice. Rats housed at thermoneutrality had a significantly elevated serum glucose area under the curve (p < 0.05) and reduced glucose-mediated insulin secretion. In contrast, rats at thermoneutrality treated with (-)-epicatechin had improved glucose tolerance and increased insulin secretion (p < 0.05). Insulin tolerance tests revealed no differences in insulin sensitivity in any of the four groups. Pancreatic immunohistochemistry staining showed significantly greater islet insulin positive cells in animals housed at thermoneutrality. In conclusion, (-)-epicatechin improved carbohydrate tolerance via increased insulin secretion in response to glucose challenge without a change in insulin sensitivity.
Investigating medicinal plants for therapeutic potential has led to medications such as metformin (from *Galega officinalis*), the most commonly prescribed medication for diabetes around the world. Diabetes and its complications pose a global threat to health, and innovations using insights from medicinal plants may provide inexpensive and effective treatment options. Flavonoids are secondary compounds found in plants that are largely responsible for flower and plant color. They are among the most diverse bioactive plant compounds in human-consumed foods. Flavonoids, and flavonoid-rich foods, have been shown to promote insulin secretion, potentiate insulin-stimulated glucose uptake, and moderate glucose-stimulated insulin secretion via the PI3K/Akt/ mammalian target of rapamycin (mTOR) pathway [2–5]. Of these compounds, (−)-epicatechin (EPICAT), found primarily in chocolate (*Theobroma cacao* L. Sterculiaceae), is highly bioactive and well studied for its support of vasodilation, cardiovascular health, mitochondrial function, and antioxidant activity [6–9].

EPICAT has shown efficacy in the treatment of metabolic conditions, including diabetes [10–14]. In a clinical trial in healthy subjects, EPICAT supplementation decreased fasting insulin and improved insulin sensitivity, but did not impact fasting blood glucose concentrations [10]. Insulin sensitivity improvements, as well as pancreatic β-cell function, improved erythrocyte function, and beneficial impacts on blood pressure parameters under stress, were also observed in clinical trials with dark chocolate [15, 16] containing EPICAT [11]. *In vivo* studies further report that EPICAT alone, cacao liquor or cocoa extract treatment, resulted in lower glucose concentrations [12–14] and alleviation of oxidative stress, cellular damage, and mitochondrial dysfunction in a model of myocardial infarction [17–19]. Most clinical trials have focused on dark chocolate and cocoa mixtures. These mixtures contain EPICAT, but detailed studies on this compound in isolation are still needed to determine specific responses. In tandem, animal models are crucial to further elucidate EPICAT’s bioactive mechanisms. Rat models of diabetes and metabolic syndrome do not precisely mimic human diabetes, type 2 diabetes, but can offer mechanistic insights in response to intervention.

Thermoneutrality (TN) refers to an environmental temperature where caloric intake is not used to maintain body temperature homeostasis. Human TN is between 14.8 °C and 24 °C [20], thus animal research environments are kept at room temperature (RT), which is comfortable to human workers. However, TN for rats is much higher at 30 °C [21, 22], prompting debate regarding optimal housing temperature for rodent research of interest to human physiology [23, 24]. Several recent studies in mice have shown that TN housing is well suited for translational comparison to humans, including similar energy expenditure to basal metabolic rate ratio [23], a cardiovascular profile more aligned with that of humans [25], induction of high-fat diet-induced diabetes [26, 27], and general differences in cardiovascular parameters of both rats and mice as compared with RT housing [28, 29]. These studies highlight that TN is likely the most translational environment for rodent biomedical research; however, little is known regarding the impact of TN on rat carbohydrate physiology.

We hypothesized that housing male Wistar rats at their TN temperature (30 °C) would result in impaired glucose tolerance and insulin resistance, verifying a model of metabolic syndrome and diabetes in which to test potential therapeutics. Further, we hypothesized that EPICAT treatment would improve metabolic profiles.

**Results**

**Metabolic parameters**

Body temperature was taken superficially, and an elevated temperature was achieved in those housed at thermoneutral conditions compared with those housed at room temperature (30.4 ± 0.1 °C vs. 27.4 ± 0.1 °C; p < 0.001). Biometric parameters of the animals were assessed at 1 week and 16 weeks of the study. Rat weight was significantly lower in those housed at TN compared with those at RT after 16 weeks (p < 0.05) (▶ Table 1). There was an effect approaching significance on the interaction of housing temperature, EPICAT, and time (p < 0.08) (▶ Table 1), with those at TN treated with EPICAT having a higher weight at 16 weeks than those at RT (▶ Table 1). There was a significant interaction effect of EPICAT and time (p < 0.05) (▶ Table 1), as well as EPICAT, time, and TN (p < 0.05) (▶ Table 1) on fasting glucose concentrations, resulting in lower concentrations in animals treated with EPICAT at RT compared with TN after 16 weeks. There were also significant effects of both EPICAT and TN on fasting insulin concentrations (p < 0.001 for both) (▶ Table 1) as well as a significant interaction of time and TN (p < 0.05) (▶ Table 1). Animals treated with EPICAT had higher insulin concentrations compared with those given vehicle, and insulin was elevated to a greater degree in those at RT (p < 0.001) (▶ Table 1). Animals housed at TN consumed significantly less food after 16 weeks than those housed at RT (p < 0.001) (▶ Table 1). There was also a significant interaction between temperature and EPICAT on food consumption, resulting in those treated with EPICAT at TN consuming more than those treated with vehicle (p < 0.05) (▶ Table 1).

(−)-Epicatechin treatment resulted in lower glucose area under the curve during intraperitoneal glucose tolerance test

Fasted animals were injected with glucose, and glucose concentrations were measured at timed intervals during a 2-h period. We observed a significant effect of EPICAT (p < 0.01), TN (p < 0.05), and interaction of time and TN on glucose area under the curve (AUC) (p < 0.01) (▶ Fig. 1). Specifically, animals treated with EPICAT showed a significantly less glucose AUC after 16 weeks compared with other groups (p < 0.01) (▶ Fig. 1). Glucose concentrations at timepoints 15, 30, 45, and 60 min were significantly different (p < 0.05 for all) (▶ Fig. 1), and animals at RT treated with EPICAT had significantly lower glucose concentrations compared to either RT or TN animals (p < 0.05 for all) (▶ Fig. 1). TN animals treated with EPICAT had a lower glucose AUC than those treated with vehicle (▶ Fig. 1).

**Insulin concentrations during intraperitoneal glucose tolerance test under thermoneutrality and (−)-epicatechin-treated conditions**

Plasma insulin concentrations and AUC were assessed during the intraperitoneal glucose tolerance test (IP-GTT) at 1 week (▶ Table 2) and 16 weeks (▶ Fig. 2). No differences were observed after
1 week of TN exposure for glucose or insulin with IP-GTT (▶Table 2). After 16 weeks of the study, there was a significant effect of TN housing on insulin AUC, resulting in an elevated AUC in those animals (p < 0.05) (▶Fig. 2). We also observed a significantly greater AUC of 253.5 ± 63.5 in EPICAT-treated animals at TN compared with 110.1 ± 20.8 in EPICAT-treated animals at RT (▶Fig. 2).

**Insulin tolerance was not impacted by either thermoneutrality housing or (-)-epicatechin treatment**

Fasted animals were injected with insulin, and glucose concentrations were measured at time intervals during a 2-h period. There were no significant effects or interactions of TN housing or EPICAT treatment on insulin sensitivity after 16 weeks (▶Fig. 3).

**Thermoneutrality housing increased islet insulin-positive cells and pancreata of (-)-epicatechin-treated animals had less insulin**

Insulin positive cells per islet were analyzed as a percentage of total islet area (▶Fig. 4a). Animals housed at TN had significantly greater insulin-positive cells per islet than those housed at RT (p < 0.05) (▶Fig. 4a). EPICAT-treated animals had significantly less insulin present in the pancreas than vehicle-treated animals, regardless of housing temperature, as expressed as insulin per total tissue area (p < 0.01) (▶Fig. 4b). There was no difference between control animals’ amount of pancreatic insulin (▶Fig. 4b).
In this study, we observed a glucose-lowering response to EPICAT treatment in both RT and TN rats. Further, animals housed at TN conditions showed significantly worse glucose tolerance and lower insulin secretion in response to IP-GTT. Animals treated with EPICAT showed higher fasting insulin concentrations and improved glucose tolerance compared with controls. EPICAT treatment significantly stimulated insulin secretion in those housed at TN. Fasting insulin concentrations were higher in the RT EPICAT-treated rodents, whereas insulin secretion was not different with IP-GTT, likely related to the absence of a significant spike in glucose with IP-GTT. In contrast to prior reports [11], we observed that insulin tolerance was not impacted by either housing temperature or EPICAT treatment. Taken together, these findings are consistent with a primary effect of insulin secretion as the media-

### Table 2

Glucose concentrations for the IP-GTT group (mg/dL) (a), concurrent insulin concentrations (ng/mL) during an IP-GTT (b), and glucose concentrations during an IP-ITT (c) at 1 week of the study. Results at 16 weeks of the study for IP-GTT and IP-ITT for all groups are presented in Figs. 1–3.

| Group   | 0 min | 15 min | 30 min | 45 min | 60 min | 120 min | AUC     |
|---------|-------|--------|--------|--------|--------|---------|---------|
| RT      | 85.4 ± 2.3 | 180.3 ± 6.5 | 125.8 ± 5.1 | 101.6 ± 2.7 | 102.1 ± 3.3 | 85.6 ± 2.9 | 3546.6 ± 283.2 |
| TN      | 85.9 ± 1.7 | 206.9 ± 10.3 | 125.4 ± 4.6 | 92.5 ± 4.7 | 98.8 ± 4.3 | 85.2 ± 2.2 | 3615.0 ± 361.8 |

| Group   | 0 min | 15 min | 30 min | 45 min | 60 min | 120 min | AUC     |
|---------|-------|--------|--------|--------|--------|---------|---------|
| RT      | 0.671 ± 0.085 | 2.134 ± 0.268 | 1.161 ± 0.110 | 0.843 ± 0.099 | 0.682 ± 0.105 | 0.927 ± 0.099 | 45.8 ± 8.6 |
| TN      | 0.611 ± 0.113 | 2.350 ± 0.360 | 1.170 ± 0.188 | 0.944 ± 0.194 | 0.844 ± 0.109 | 0.752 ± 0.102 | 62.1 ± 15.2 |

| Group   | 0 min | 15 min | 30 min | 45 min | 60 min | 120 min | AUC     |
|---------|-------|--------|--------|--------|--------|---------|---------|
| RT      | 88.2 ± 3.5 | 69.0 ± 2.6 | 51.7 ± 2.6 | 41.7 ± 2.3 | 40.8 ± 3.4 | 62.0 ± 5.1 | 4737.7 ± 511.7 |
| TN      | 85.2 ± 2.5 | 64.4 ± 4.0 | 42.2 ± 2.4 | 36.2 ± 2.3 | 34.7 ± 2.0 | 61.7 ± 4.3 | 4936.4 ± 329.6 |

*P < 0.05 temperature, * EPICAT, *time × temperature, *time × EPICAT, *time × EPICAT × temperature effects; †p < 0.08 time × temperature × EPICAT, three-way ANOVA, mean ± SEM, n = 8.

**Discussion**

In this study, we observed a glucose-lowering response to EPICAT treatment in both RT and TN rats. Further, animals housed at TN conditions showed significantly worse glucose tolerance and lower insulin secretion in response to IP-GTT. Animals treated with EPICAT showed higher fasting insulin concentrations and improved glucose tolerance compared with controls. EPICAT treatment significantly stimulated insulin secretion in those housed at TN. Fasting insulin concentrations were higher in the RT EPICAT-treated rodents, whereas insulin secretion was not different with IP-GTT, likely related to the absence of a significant spike in glucose with IP-GTT. In contrast to prior reports [11], we observed that insulin tolerance was not impacted by either housing temperature or EPICAT treatment. Taken together, these findings are consistent with a primary effect of insulin secretion as the media-

**Fig. 2** Concurrent insulin concentrations during IP-GTT. Plasma insulin concentrations were measured during IP-GTT, and area under the curve (AUC) was determined for each group at 16 weeks. *P < 0.05 for effect of temperature, two-way ANOVA or mixed-effects model. Data are presented as the mean ± SEM, n = 8.
tor of the glucose-lowering effect of EPICAT. These data build upon reported studies on EPICAT’s glucose-lowering bioactivity [10, 12–14, 30].

Glucose intolerance was present in our animals housed at TN, as measured by IP-GTT. Concurrent measurements of insulin secretion with the IP-GTT suggest that insulin secreted by TN-housed animals is inadequate, as we observed glucose intolerance without a change in insulin sensitivity. Treating animals housed at both RT and TN conditions with EPICAT resulted in improved glucose tolerance, most dramatically in animals housed at RT, aligning with previous reports [10, 12–14, 31].

To address impacts of housing temperature and EPICAT treatment on insulin secretion, we investigated pancreatic islets. The amount of insulin per islet was significantly elevated in those housed at TN conditions, regardless of EPICAT treatment, consistent with pancreatic compensation in the context of impaired glucose tolerance in these animals. Impaired or compensatory insulin secretion and production is common in metabolic syndrome and glucose tolerance in these animals. Impaired or compensatory insulin secretion. Previous reports on islet compensation show a larger islet area per total pancreas regardless of housing environment. Taken together, these pancreatic analyses may point to EPICAT’s ability to augment efficiency of glucose-mediated insulin secretion. Previous reports on islet compensation show a larger islet area with less insulin staining per islet [33]. Our observation warrants further study as we noted improved insulin secretion without detecting a change in insulin staining per islet with EPICAT. The finding of augmented insulin secretion with 15 days of EPICAT treatment is most consistent with improved glucose-mediated insulin response. Our study shows that EPICAT improved insulin secretion in tandem with a glucose challenge, improving the match of insulin supply with glucose demand. Overall, these results agree with in vitro studies showing that EPICAT stimulates insulin secretion [34–36].

There are several mechanisms potentially explaining our results that warrant future investigation. For example, EPICAT is reported to increase circulating glucagon like peptide – 1 (GLP-1) plasma concentrations [37], an incretin that augments glucose-mediated insulin secretion. Other reports state that EPICAT can act on cellular signaling in the beta cell via the CaMKII pathway upstream of insulin secretion [35], and/or the stimulation of tissue-localized mitochondrial respiration (to augment beta cell glucose sensing) [38]. Most notably, EPICAT may be acting on various forms of nitric oxide synthase (NOS). Inducible/inflammatory NOS (iNOS) blunts insulin secretion. Elevated pancreatic iNOS, induced by inflammatory processes, suppresses insulin secretion [39–41]. EPICAT has been shown to decrease excess iNOS [42, 43], resulting in improved insulin secretion [43]. Endothelial NOS (eNOS) is activated by insulin signaling through the PI3K/Akt/eNOS cascade [44]; many studies show that EPICAT stimulates eNOS [7, 9, 45, 46]. In light of this previous work, our data could be explained by a model by which EPICAT may stimulate eNOS or suppress pancreatic iNOS, resulting in robust insulin secretion in response to glucose concentrations further normalizing postprandial carbohydrate dynamics. However, we showed no effect of EPICAT on insulin per islet, consistent with EPICAT acting on the secretion process itself in response to glucose rather than on β-cell function or adaptation. Future studies will address these and other mechanisms.

Intriguingly, insulin sensitivity in the intraperitoneal insulin tolerance test (IP-ITT) was unaffected by either housing temperature or EPICAT treatment. Although a study reported similar data in a clinical trial [47], this is unexpected in light of the significant effects of both variables on glucose tolerance and islet insulin, and also in contrast to other previous clinical studies [10, 48, 49]. We interpret these results as pointing to adaptation of glucose handling independent of the insulin signaling cascade involving cellular glucose uptake and/or β-cell function. EPICAT clearly modulates insulin secretion and perhaps glucose uptake at the cellular level, independent of impacts on insulin sensitivity.

We observed divergent impacts of EPICAT on fasting glucose concentrations, resulting in lower concentrations in animals housed at RT and higher concentrations in those housed at TN. As there were significant interactions between EPICAT, time, and temperature on this parameter, it follows that the interplay of the
variables is impacting glucose concentrations. Although TN housing caused lower fasting insulin concentrations overall, EPICAT treatment resulted in higher concentrations compared with vehicle controls, agreeing with our other data showing more efficient insulin production and improved glucose tolerance.

This study has several limitations. Although our dosage and duration of EPICAT treatment was devised based on previous successful studies [12, 50, 51], 15 days of this compound may not be enough time to impact insulin secretion and insulin action in animals housed at TN. Other reports have addressed bioavailability by administering EPICAT in a mixture [10, 48, 49], and we used DMSO and a saline solution for solubility in oral gavage. This may have affected bioavailability, although we do report significant impacts on glucose tolerance and insulin secretion and related efficiency. Despite this, our dosage of 1 mg/kg body weight may also have been too low to perturb certain metabolic aspects in TN animals; dose responses will be considered in future studies.

Also, to definitively conclude EPICAT’s mechanism of action in insulin secretion requires further study, especially in different animal models to verify broad bioactivity. Cellular signaling upstream of insulin secretion and glucose uptake may also be important to further define EPICAT’s antidiabetic bioactivity. Although this thermoneutral animal model has been ideal for the purpose of this work, additional studies are planned. Interestingly, we showed that animals housed at their thermoneutral temperature show metabolic dysfunction. This has been reported in previous reports for rodents [23, 24, 26, 27], and is one of the reasons this housing is an emerging method towards aligning rodent models with human metabolism. The physiology behind these unique metabolic responses to thermoneutral housing is a subject of ongoing investigation in our laboratory and others.

In conclusion, we show that EPICAT lowers glucose and improves insulin response to a glucose load, with a likely modulation of pancreatic insulin production and secretion. In addition, our
study also shows that animals housed at TN consume less food, agreeing with other reports [52]. Investigating EPICAT with the use of IP-GTT concurrent insulin concentrations and islet insulin quantification is a somewhat unique approach to ascertaining botanical anti-diabetic activity. Although we are not the first to report that EPICAT stimulates insulin secretion, our study adds to the existing data in the field by utilizing a thermoneutral animal model. Housing rats at their thermoneutral temperatures provides a novel animal paradigm of metabolic dysfunction easy to establish and prime for screening specific bioactivity from natural products and functional foods. Our results have broad implications for human health, as diabetes is prevalent around the world [1]. Importantly, EPICAT is already sold and consumed as a supplement as well as a component of highly popular foods, making it immediately applicable in several forms. Future studies will focus on a mechanistic evaluation of EPICAT’s impact on islet function and insulin secretion.

Methods and Materials

Reagents

Pharmaceutical grade glucose and PBS, and EDTA are from Sigma-Aldrich. DMSO, sodium chloride, and bovine serum albumin were purchased from Fisher Scientific. Insulin (Humulin R) was manufactured by Eli Lilly and Company. EPICAT was procured from Cayman Chemical. Unconjugated FLEX Polyclonal Guinea Pig Anti-Insulin antibody was used at a 1:5 dilution in 2% Normal Donkey Serum in 1×PBST. Alexa Fluor 488 AffiniPure Donkey Anti-Guinea Pig IgG (H+L) was diluted 1:500 in 2% NDS/PBST. DAPI was diluted 1:1000 in 1×PBST. Prolong Gold Antifade Mountant was purchased from Thermo Fisher Scientific.

In vivo experiments

The animal study protocol was approved by the Institutional Animal Care and Use Committee of the RMR VA Medical Center, protocol number 2CD2013R, approved July 14, 2020. Animals (male Wistar rats, 5 weeks old, procured from Charles River Laboratories, Inc.), kept at 2 animals per cage, were housed at either RT (22 °C) or TN (29–30 °C) from the time they arrived at the facility. As quarantine is 1 week, all measurements were taken 1 week after initial housing. Animals were fed a customized diet containing 13% kcal fat (LFD) (Envigo [Teklad]) for 16 weeks, and randomized into 4 groups, with n = 8 in each group: RT + vehicle, RT + EPICAT, TN + vehicle, and TN + EPICAT. EPICAT solution was provided at 1 mg/kg body weight by diluting the specific dosage from a 15 mg/mL stock in 50:50 DMSO:PBS (vehicle) up to 0.046 mL for a final DMSO dosage of 1.58% of the solution. For a final gavage volume of 1.5 mL total, the dosage or vehicle was diluted in 1.454 mL of PBS. During the final 15 days, animals were gavaged test solutions once per day before noon. Blood (approximately 50 μL) from the tail vein was collected in 0.5 M EDTA and spun at 12,000 g for 10 min at 4 °C. Plasma was extracted and stored at −80 °C. Fasting blood (6 h), also from the tail vein, was taken at the beginning and end of the study and fed blood from the tail vein was taken biweekly for glucose and insulin concentrations. Body weight and food consumption were measured weekly.

Endpoint parameters were taken at sacrifice, and all animals were euthanized in the morning following ad libitum food consumption. Euthanasia was conducted via isoflurane gas administration, along with consistent oxygen. This method of euthanasia complies with IACUC requirements and was formally approved in the protocol listed above.

Insulin and glucose intraperitoneal tolerance tests

Insulin tolerance testing (ITT) was done at 1 and 16 weeks of the study, following a 6-hour fast, by interperitoneal injection of 1 U·kg⁻¹ body weight of insulin [53]. Blood glucose concentrations were sampled at 0, 15, 30, 45, 60, and 120 min post-injection. Glucose tolerance testing (GTT) followed the same protocol using 1.5 g·kg⁻¹ body weight of glucose, injected intraperitoneally, and was separated from ITT testing by 4 days. Fasting glucose and insulin concentrations were taken as the 0-minute blood collection during GTT. Baseline concentrations were subtracted for AUC analyses.

Pancreatic staining

The pancreas was collected from each animal after sacrifice and processed and analyzed the same way for each animal. Each pancreas was fixed for 24 hours in 10% formalin, then rinsed 3 times in 70% EtOH. These tissue samples were then processed in paraffin overnight using a Histocore Pearl instrument and embedded in paraffin using a Tissue Tek II instrument (Sakara Finetek). Each tissue mold was then sectioned into 5 μm sections using a microscope and positioned on positively charged slides. The slides were incubated overnight at 65 °C. Slides were incubated in xylene 2 times for 10 min each, 100% EtOH 2 times for 10 min each, 95% EtOH for 3 min, 70% EtOH for 3 min, 50% EtOH for 3 min, rinsed in DI H₂O twice, and rehydrated in 1×PBST for 10 min. Each tissue section was blocked using 2% NDS in 1×PBST for 30 min at RT. After removing the blocking solution, unconjugated FLEX Polyclonal Guinea Pig Anti-Insulin antibody (1:5) was applied and incubated overnight in 4 °C covered with paraffilm. The next day, the paraffilm was removed and each slide was washed in 1×PBST 4 times for 5 min each. Alexa Fluor 488 AffiniPure Donkey Anti-Guinea Pig IgG (H+L) (1:500) was applied and incubated for 1 h at RT, protected from light and covered with paraffilm. Paraffilm was removed and slides washed again 4 times in 1×PBST for 5 min each. DAPI was applied to each tissue section and incubated for 15 min at RT, protected from light. The slides were washed 2 times in 1×PBST and mounted using Prolong Gold mounting solution and a glass coverslip. This protocol is very similar to that previously described [54]. Each tissue section was imaged using a Keyence BX-Z800 fluorescence microscope with a DAPI and FITC lasers and 20× zoom lens. Animals treated with EPICAT were compared with pooled controls of animals housed at RT and TN to allow for a robust statistical analysis.

Islet parameter assessment

A custom Matlab M-file was written to analyze the number of islets per section of pancreas as assessed by DAPI staining. The software reads an image into Matlab as an RGB image and then is made into a binary image based on fluorescence intensity. Otsu’s method was used to choose a threshold of pixel intensity that
minimizes the intraclass variance of the thresholded black and white pixels. The “binarization” uses a 256-bin image histogram to compute Otsu’s threshold. The code begins by removing intensity artifacts from the green layer of the image (insulin positive) by eliminating items less than 15 pixels in size. The code then fills in any gaps within identified bodies larger than 15 pixels. This step is used to account for intensity gaps in the islet and helps to accurately measure islet perimeter and area. The Matlab function “bwboundaries” is then employed to find all boundaries in the image and aids in distinguishing true islets from artifacts by utilizing islet morphology. The area and perimeter of the islets are determined as part of the “bwboundaries” function. These two parameters are used in a metric calculation to determine islet morphology based on a sliding scale where 1 represents an islet and 0 represents an artifact in the image. The metric uses a threshold of 0.98 to identify true islets. Finally, the islets are evaluated by another threshold that measures size and islet morphology metric score concurrently and islets that do not meet a user defined value are removed. The remaining islets are exported to an Excel file with the total islet count, area of each islet, and metric score listed.

Fluorescence imaging assessment

A second custom Matlab M-file was used to identify the FITC-positive (insulin) regions in the pancreas section (DAPI positive). The script asks the user to define an area of background fluorescence on the green layer (insulin) in the image. The mean intensity of this defined region is then used to background subtract the entire green layer; values less than zero are assigned a zero value. The user then selects the entire slice of tissue in the blue layer (DAPI) and a binary mask is generated. The binary mask is then applied to the green layer of the image. Values inside the region of interest retain their fluorescence intensity and items outside are assigned a zero value. The insulin layer was then binarized into pixels with a fluorescence intensity (1) or areas without (0). The insulin-positive areas were summed and divided by the area of the user-defined slice and multiplied by 100. The percentage of insulin-positive area to DAPI-positive area was exported to the same Excel file.

Statistical analysis

To analyze data with time/dose along with EPICAT and temperature, we employed a repeated measures ANOVA for the variable temperature and a mixed-effects model. For data without a time or dose component, we employed a repeated measures ANOVA, along with a post hoc analysis within ANOVA. A p value of less than 0.05 was used as the cutoff for statistical significance in all tests. A p value of equal or less than 0.08 was considered indicative of data trends approaching significance and thus reported.

Contributors’ Statement

ACK and JEBR generated ideas, wrote the manuscript, housed the project and provided oversight. ACK, JHC, LAK, SEH helped with writing the manuscript, conducted experiments, generated data and performed data analysis. MMH, GBP, and DGR generated data and performed data analysis.

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Conflict of Interest

The authors declare that they have no conflict of interest.

References

[1] WHO. Diabetes fact sheet. News Room 2021. Accessed November 5, 2021 at: https://www.who.int/news-room/fact-sheets/detail/diabetes
[2] Santangelo C, Zicari A, Mandosi E, Scazzocchio B, Mari E, Morano S, Masella R. Could gestational diabetes mellitus be managed through dietary bioactive compounds? Current knowledge and future perspectives. Br J Nutr 2016; 115: 1129–1144
[3] Oh YS, Jun HS. Role of bioactive food components in diabetes prevention: Effects on Beta-cell function and preservation. Nutr Metab Insights 2014; 7: 51–59
[4] Hanhineva K, Torronen R, Bondia-Pons I, Pekkinen J, Kolehmainen M, Mykkanen H, Poutanen K. Impact of dietary polyphenols on carbohydrate metabolism. Int J Mol Sci 2010; 11: 1365–1402
[5] Martin MA, Ramos S. Dietary flavonoids and insulin signaling in diabetes and obesity. Cells 2021; 10: 1474
[6] Keller A, Hull SE, Elajaili H, Johnston A, Knaub LA, Chun JH, Walker L, Nozik-Grayck E, Reusch JEB. (-)-Epicatechin modulates mitochondrial redox in vascular cell models of oxidative stress. Oxid Med Cell Longev 2020; 2020: 6392629
[7] Galleano M, Bernatova I, Puzserova A, Balis P, Sestakova N, Pechanova O, Fraga CG. (-)-Epicatechin reduces blood pressure and improves vasorelaxation in spontaneously hypertensive rats by NO-mediated mechanisms. IUBMB Life 2013; 65: 710–715
[8] Tanabe K, Tamura Y, Lanasa MA, Miyazaki M, Suzuki N, Sato W, Maesinha Y, Schreiner GF, Villarreal FJ, Johnson RJ, Nakagawa T. Epicatechin limits renal injury by mitochondrial protection in cisplatin nephropathy. Am J Physiol Renal Physiol 2012; 303: F1264–F1274
[9] Litterio MC, Vazquez Prieto MA, Adamo AM, Elesgaray R, Oteiza PI, Galleano M, Fraga CG. (-)-Epicatechin reduces blood pressure increase in high-fructose-fed rats: Effects on the determinants of nitric oxide bioavailability. J Nutr Biochem 2015; 26: 745–751
[10] Dower JL, Geleijnse JM, Gijbers L, Zock PL, Kromhout D, Holman PC. Effects of the pure flavonoids epicatechin and quercetin on vascular function and cardiometabolic health: A randomized, double-blind, placebo-controlled, crossover trial. Am J Clin Nutr 2015; 101: 914–921
[11] Grassi D, Lippi C, Nezzone S, Desideri G, Ferri C. Short-term administration of dark chocolate is followed by a significant increase in insulin sensitivity and a decrease in blood pressure in healthy persons. Am J Clin Nutr 2005; 81: 611–614
[12] Gutierrez-Salmean G, Ortiz-Vilchis P, Vacaseydel CM, Garduno-Siciliano L, Chamorro-Cevallos G, Meaney E, Villafana S, Villarreal F, Ceballos G,
Ramirez-Sanchez I. Effects of (−)-epicatechin on a diet-induced rat model of cardiometabolic risk factors. Eur J Pharmacol 2014; 728: 24–30

[13] Ruzaidi A, Amin I, Nawaiyah AG, Hamid M, Faizul HA. The effect of Malaysian cocoa extract on glucose levels and lipid profiles in diabetic rats. J Ethnopharmacol 2005; 98: 55–60

[14] Tomaru M, Takano H, Yasuda A, Inoue K, Yanagisawa R, Ohwata T, Uematsu H. Dietary supplementation with cacao liquor proanthocyanidins prevents elevation of blood glucose levels in diabetic obese mice. Nutrition 2007; 23: 351–355

[15] Radosinska J, Horvathova M, Frimmel K, Muchova J, Vidosovicova M, Vazan R, Bernatova I. Acute dark chocolate ingestion is beneficial for hemodynamics via enhancement of erythrocyte deformability in healthy humans. Nutr Res 2017; 39: 69–75

[16] Regecova V, Jurkovicova J, Babjakova J, Bernatova I. The effect of a single dose of dark chocolate on cardiovascular parameters and their reactivity to mental stress. J Am Coll Nutr 2020; 39: 414–421

[17] Prince PS. A biochemical, electrocardiographic, electrophoretic, histopathological and in vitro study on the protective effects of (−)-epicatechin in isoproterenol-induced myocardial infarcted rats. Eur J Pharmacol 2011; 671: 95–101

[18] Prince PS. (−) Epicatechin prevents alterations in lysosomal glycohydrodases, cathepsins and reduces myocardial fat size in isoproterenol-induced myocardial infarcted rats. Eur J Pharmacol 2013; 706: 63–69

[19] Stanely Mainzen Prince P. (−) Epicatechin attenuates mitochondrial damage by enhancing mitochondrial multi-marker enzymes, adenosine triphosphate and lowering calcium in isoproterenol induced myocardial infarcted rats. Food Chem Toxicol 2013; 53: 409–416

[20] Kingma BR, Frijns AJ, Schellen L, van Marken Lichtenbelt WD. Beyond the zone of thermal neutrality. J Appl Physiol (1985) 2002; 92: 2667–2679

[21] Poole S, Stephenson JD. Body temperature regulation and thermoneutrality in rats. Q J Exp Physiol Cogn Med Sci 1977; 62: 143–149

[22] Fischer AW, Cannon B, Nedergaard J. Optimal housing temperatures for mice to mimic the thermal environment of humans: An experimental study. Mol Metab 2018; 7: 161–170

[23] Keijer J, Li M, Speakman JR. What is the best housing environment to translate mouse experiments to humans? Mol Metab 2019; 25: 168–176

[24] Overton JM. Phenotyping small animals as models for the human metabolic syndrome: thermoneutrality matters. Int J Obes (Lond) 2010; 34: S53–S58

[25] Steimer K, Kotzebek P, Zani F, Bauer M, Neff C, Muller TD, Pflugler PT, Seleye RJ, Divanovic S. Thermoneutral housing is a critical factor for immune function and diet-induced obesity in CS7BL/6 nude mice. Int J Obes (Lond) 2015; 39: 791–797

[26] Canehan K, Chawla A. Warming the use to model human diseases. Nat Rev Endocrinol 2017; 13: 458–465

[27] Swoap SJ, Overton JM, Garber G. Effect of ambient temperature on cardiovascular parameters in rats and mice: a comparative approach. Am J Physiol Regul Integr Comp Physiol 2004; 287: R391–R396

[28] Maloney SK, Fuller A, Mitchell D, Gordon C, Overton JM. Translating animal model research: does it matter that our rodents are cold? Physiology (Bethesda) 2014; 29: 413–420

[29] Gutierrez-Salmean G, Ortíz-Vilchis P, Vacasaydel CM, Rubio-Gayoso I, Meaney E, Villarreal F, Ramirez-Sanchez I, Ceballos G. Acute effects of an oral supplement of (−)-epicatechin on postprandial fat and carbohydrate metabolism in normal and overweight subjects. Food Funct 2014; 5: S21–S27

[30] Mahler RJ. The relationship between the hyperplastic pancreatic islet and insulin insensitivity in obesity. Acta Diabetol Lat 1981; 18: 1–17

[31] Huang HH, Novikova L, Williams SJ, Smirnova IV, Stehno-Bittel L. Low insulin content of large rat population is present in situ and in isolated islets. Idts 2011; 3: 6–13

[32] Bitner BF, Ray JD, Nerenberg KB, Heringa JA, Tauber JA, Johnson DK, Tellez Freitas CM, Fauvet R, Allen ME, Thomson AH, Weber KS, McMillan RP, Hulver MW, Brown DA, Tessem JS, Neillson AP. Common gut microbiota metabolites of dietary flavonoids exert potent protective activities in beta-cells and skeletal muscle cells. J Nutr Biochem 2018; 62: 95–107

[33] Yang K, Chan CB. Epicatechin potentiation of glucose-stimulated insulin secretion in INS-1 cells is not dependent on its antioxidant activity. Acta Pharmocol Sin 2018; 39: 893–902

[34] Hiś CS, Howell SL. Effects of epicatechin on rat islets of Langerhans. Diabetes 1984; 33: 291–296

[35] Cremonini E, Daveri E, Mastaloudis A, Oteiza PI. (−)-Epicatechin and anthocyanins modulate GLUT-1: evidence from C57BL/6j mice and GLUT2 tagged cells. J Nutr 2021; 151: 1497–1506

[36] Rowley Tj 4th, Bitner BF, Ray JD, Lathen DR, Smithson AT, Dollon BW, Plowman CJ, Bikman BT, Hansen JM, Dorenkott MR, Goodrich KM, Ye L, O’Keeffe SF, Neillson AP, Tessem JS. Monomeric cocoa catechins enhance beta-cell function by increasing mitochondrial respiration. J Nutr Biochem 2017; 49: 30–41

[37] Mussa BM, Srivastava A, Mohammed AK, Verberne AJM. Nitric oxide interacts with cholinicoceptors to modulate insulin secretion by pancreatic beta cells. Pflugers Arch 2020; 472: 1469–1480

[38] Shahrazi KS, Karbalaei N, Nematí M. Improving effect of combined inorganic nitrate and nitric oxide synthase inhibitor on pancreatic oxidative stress and impaired insulin secretion in streptozotocin-induced diabetic rats. J Diabetes Metab Disord 2020; 19: 353–362

[39] Bahadoran Z, Mirrmanip P, Ghassemi A. Role of nitric oxide in insulin secretion and glucose metabolism. Trends Endocrinol Metab 2020; 31: 118–130

[40] Mohamed RH, Karam RA, Amer MG. Epicatechin attenuates doxorubicin-induced brain toxicity: Critical role of TNF-alpha, iNOS and NF-kappaB. Brain Res Bull 2011; 86: 22–28

[41] Kim MJ, Ryu GR, Kang JH, Sim SS, Min DS, Rhie DJ, Hahn SJ, Jeong IK, Hong KJ, Kim MS, Jo YH. Inhibitory effects of epicatechin on interleukin-1beta-induced inducible nitric oxide synthase expression in NIH3T3 cells and rat pancreatic islets by down-regulation of NF-kappab activation. Biochem Pharmacol 2004; 68: 1775–1785

[42] Chebli S, Samsonov AP, Chebli S, Vazquez AB, Kashfi K. Regulation of carbohydrate metabolism by nitric oxide and hydrogen sulfide: Implications in diabetes. Biochem Pharmacol 2020; 176: 113819

[43] Ramirez-Sanchez I, Maya L, Ceballos G, Villarreal F. (−)-Epicatechin activation of endothelial cell endothelial nitric oxide synthase, nitric oxide, and related signaling pathways. Hypertension 2010; 55: 1398–1405

[44] Taub PR, Ramirez-Sanchez I, Ciarraldi TP, Perkins G, Murphy AN, Naviaux R, Hogan M, Maisel AS, Henry RR, Ceballos G, Villarreal F. Alterations in skeletal muscle indicators of mitochondrial structure and biogenesis in patients with type 2 diabetes and heart failure: Effects of epicatechin rich cocoa. Clin Transl Sci 2012; 5: 43–47

[45] Stote KS, Clevendine BA, Novotny JA, Henderson T, Radecki SV, Baer DJ. Effect of cocoa and green tea on biomarkers of glucose regulation, oxidative stress, inflammation and hemostasis in obese adults at risk for insulin resistance. Eur J Clin Nutr 2012; 66: 1153–1159
[48] Davison K, Coates AM, Buckley JD, Howe PR. Effect of cocoa flavanols and exercise on cardiometabolic risk factors in overweight and obese subjects. Int J Obes (Lond) 2008; 32: 1289–1296

[49] Desideri G, Kwik-Uribe C, Grassi D, Necozione S, Chiadoni L, Mastroiacovo D, Raffaele A, Ferri L, Boccale R, Lechiara MC, Marini C, Ferri C. Benefits in cognitive function, blood pressure, and insulin resistance through cocoa flavanol consumption in elderly subjects with mild cognitive impairment: The Cocoa, Cognition, and Aging (CoCoA) study. Hypertension 2012; 60: 794–801

[50] Ramirez-Sanchez I, Taub PR, Ciaraldi TP, Nogueira L, Coe T, Perkins G, Hogan M, Maisel AS, Henry RR, Ceballos G, Villarreal F. (-)-Epicatechin rich cocoa mediated modulation of oxidative stress regulators in skeletal muscle of heart failure and type 2 diabetes patients. Int J Cardiol 2013; 168: 3982–3990

[51] Ramirez-Sanchez I, De los Santos S, Gonzalez-Basurto S, Canto P, Mendoza-Lorenzo P, Palma-Flores C, Ceballos-Reyes G, Villarreal F, Zentella-Dehesa A, Coral-Vazquez R. (-)-Epicatechin improves mitochondrial-related protein levels and ameliorates oxidative stress in dystrophic delta-sarcoglycan null mouse striated muscle. FEBS J 2014; 281: 5567–5580

[52] Kaiyala KJ, Morton GJ, Thaler JP, Meek TH, Tylee T, Ogimoto K, Wisse BE. Acutely decreased thermoregulatory energy expenditure or decreased activity energy expenditure both acutely reduce food intake in mice. PloS One 2012; 7: e41473

[53] Keller AC, He K, Brissallantes AM, Konnelly EJ. A characterized saponin-rich fraction of Momordica charantia shows antidiabetic activity in C57BLK/6 mice fed a high fat diet. Phytomedicine Plus 2021; 1: 100134

[54] Ramirez DG, Abenojar E, Hernandez C, Lorberbaum DS, Papaizan LA, Passman S, Pham V, Enser AA, Benninger RKP. Contrast-enhanced ultrasound with sub-micron sized contrast agents detects insulinitis in mouse models of type 1 diabetes. Nat Commun 2020; 11: 2238