Investigating The Effects Of Diet-Induced Pre-Diabetes On Calciotropic Hormones In Male Sprague Dawley Rats

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Research Article

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Abstract

Calcium homeostasis is disturbed emanating from altered calcitropic hormone concentrations in type 2 diabetes mellitus (T2DM), a condition preceded by pre-diabetes. Disrupted calcium homeostasis has shown to promote insulin resistance and hyperglycaemia. However, the changes to calcitropic hormones in the pre-diabetic state is not known. Hence, this study investigated the effects of diet-induced pre-diabetes on calcitropic hormones in a pre-diabetic rat model. This study also sought to determine the association of calcitropic hormones with glycated haemoglobin (HbA1c) and insulin resistance during pre-diabetes. Twelve male Sprague Dawley rats were randomly assigned into two groups (n=6, per group): pre-diabetic (PD) and non-pre-diabetic (NPD). Fasting blood glucose (FBG), insulin, HbA1c and the homeostatic model assessment for insulin resistance (HOMA-IR) in addition to urine calcium, plasma calcium, parathyroid hormone (PTH), calcitonin, vitamin D and calcitriol concentrations were analysed at week 20. Correlation analysis was performed to examine the associations of calcitropic hormones with HOMA-IR and HbA1c. The results demonstrated increased concentrations of HbA1c, FBG, insulin and HOMA-IR in the PD group by comparison to NPD. Furthermore, plasma PTH, calcitonin, urine calcium, calcitriol and vitamin D levels increased along with unchanged plasma calcium concentrations in the PD group by comparison to NPD. Plasma PTH and calcitonin levels were positively correlated with HbA1c but not with HOMA-IR in the PD group. Plasma calcitriol levels were negatively correlated with HbA1c in the PD group. These observations suggest that calcium homeostasis is disturbed in diet-induced pre-diabetes but the body compensates for the changes by inducing an increase in calcitropic hormone levels. Furthermore, pre-diabetes may promote the development of hyperglycemia in T2DM through altering calcitropic hormone levels.

Introduction

Pre-diabetes is a state that exists between normoglycaemia and T2DM, and it occurs before T2DM develops [1]. This condition is characterised by impaired fasting glucose (IFG), impaired glucose tolerance (IGT) and elevated glycated haemoglobin (HbA1c) concentrations [2]. Pre-diabetes and type 2 diabetes are linked to sedentary lifestyles and the consumption of high-fat and high-carbohydrate diets [3]. The International Diabetes Federation (IDF) estimates that by 2030 over 578 million people worldwide are expected to have diabetes, while it is further anticipated that over 470 million people would be pre-diabetic by 2050 [4]. A positive correlation has been noted between altered calcium homeostasis, abnormal blood glucose levels, insulin resistance and β-cell dysfunction [2].

Calcium homeostasis is regulated by calcitropic hormones, namely parathyroid hormone (PTH), calcitonin and 1,25-dihydroxyvitamin D3 also known as calcitriol which act on the intestine, kidney and bone [5]. Increased renal calcium reabsorption, intestine absorption and breakdown of bone allow plasma calcium levels to be conserved as a result of these interactions [6]. Several studies have shown that calcium homeostasis is disturbed in T2DM and have used calcitropic hormones as markers to study the overall calcium status of the body [7, 8]. Studies have shown changes to plasma calcium, urinary calcium and calcitropic hormone levels in T2DM individuals [9, 10]. Alterations to calcitropic hormone concentrations may occur due to pathological changes that occur to the organs which synthesis these hormones or as a compensatory response to disturbed plasma calcium levels in T2DM [11, 12]. Furthermore, studies have shown that derangements to calcitropic hormone levels may exacerbate insulin resistance in the diabetic state [6, 7]. Optimal intracellular levels of calcium are required for the adequate functioning of pancreatic β-cells as well as insulin-responsive tissue such as the liver and skeletal muscles [13]. There are various processes that are dependent on calcium in the body that would be impaired by calcium homeostasis being interrupted [13].
In our laboratory, a pre-diabetic model of the human condition of pre-diabetes was created by feeding rats a high-fat high-carbohydrate diet [14, 15]. Several investigations employing this model have found that various complications associated with T2DM begin in the pre-diabetic stage [15, 16]. In the diabetic state, the changes to calcium homeostasis and the association of calciotropic hormones with glycated haemoglobin (HbA1c) and insulin resistance have been well documented [9, 10]. However, these changes have not yet been investigated during the pre-diabetic state. Hence, this study aimed to investigate the effects of diet-induced pre-diabetes on calciotropic hormones in a pre-diabetic rat model. Furthermore, this study also sought to determine the association of calciotropic hormones with glycated haemoglobin (HbA1c) and insulin resistance in a pre-diabetic rat model.

Materials And Methods

Animals and housing

This study employed male Sprague-Dawley rats (150-180g) which were bred and housed at the University of KwaZulu-Natal Biomedical Research Unit (BRU). Male rats were selected due to their more stable hormonal profile in comparison to female subjects. The animals were kept under standard laboratory conditions, which included a constant temperature of 22 ± 2°C, a carbon dioxide (CO2) content of < 5000 p.m, a relative humidity of 55 ± 5% and illumination (12 hour light/dark cycle, lights on at 07h00). The noise level was maintained at less than 65 decibels. The animals were allowed access to food and fluids ad libitum. The Animal Research Ethics Committee of the University of KwaZulu-Natal (ETHICS#: AREC/00003627/2021) approved all animal experimentation. The animals were allowed to acclimatize to their new environment for 1 week while consuming standard rat chow and tap water before exposure to the experimental diets [14]. The procedures involving animal care followed the University of KwaZulu-Natal's institutional guidelines for animal care.

Induction of pre-diabetes

For an experimental period of 20 weeks, rats were randomly divided into two groups (n = 6, per group) and fed their respective diets. Experimental pre-diabetes was induced in the animals using a previously described protocol by Luvuno et al. 2017 [14]. To induce pre-diabetes, one group was fed a high-fat high-carbohydrate (HFHC) diet supplemented with 15% fructose enriched water (AVI Products (Pty) Ltd, Waterfall, South Africa), whereas the other group was fed a standard rat chow and tap water. The animals were evaluated for pre-diabetes after 20 weeks using the American Diabetes Association (ADA) criteria. Animals with a fasting blood glucose concentration of 5.6 to 6.9 mmol/L, oral glucose tolerance test (OGTT) 2-h glucose concentration of 7.8 to 11.0 mmol/L and a glycated haemoglobin concentration of 5.7 to 6.4% were regarded as pre-diabetic. The animals that were fed the standard diet were also tested at week 20 to confirm normoglycaemia.

Experimental design

This study comprised of two groups, namely a non-pre-diabetic (NPD) group and a pre-diabetic (PD) group (n = 6, in each group). The NPD group consisted of animals which consumed the standard rat chow for 20 weeks and did not have pre-diabetes, while the PD group consisted of animals which consumed the HFHC diet for the same number of weeks and were diagnosed with pre-diabetes.

Urine and blood collection

At the end of the experimental period, all animals were housed individually in Makrolon polycarbonate metabolic cages (Techniplats, Labotec, South Africa) for a 24-hour urine collection period. Thereafter, the urine samples were centrifuged (Eppendorf centrifuge 5403, LGBW Germany) at 1000 g for 20 minutes at 4°C. The supernatants were
then frozen at -80°C in a Bio Ultra freezer (Labotec, Umhlanga, South Africa). Thereafter, the animals were anaesthetized with Isofor (100 mg/kg) (Safeline Pharmaceuticals (Pty) Ltd, Roodeport, South Africa) for 3 minutes via a gas anaesthetic chamber (Biomedical Resource Unit, UKZN, South Africa). Blood was collected by cardiac puncture while the rats were unconscious and then injected into individual pre-cooled heparinized containers. The blood was centrifuged (Eppendorf centrifuge 5403, LGBW Germany) for 15 minutes at 4°C, 503 g. Plasma was isolated from blood and stored in a Bio Ultra freezer (Labotec, Umhlanga, South Africa) at -80°C until biochemical analysis, as previously described by Luvuno et al., 2018 [19]. Of note, plasma and urine samples were obtained from a previous study (ETHICS#: AREC/00003627/2021).

HOMA-IR index

Insulin resistance was calculated from fasting blood glucose and insulin levels using the homeostatic model assessment (HOMA) [20]. The HOMA-IR index was calculated using the HOMA2 Calculator v2.2.3 program [21]. Insulin sensitive is values < 1.0, early insulin resistance is values > 1.9, and significant insulin resistance is values > 2.9.

Biochemical analysis

An autoanalyzer (IDEXX VetLab station, Hoofddorp, Netherlands) was used to determine the concentration of calcium in the plasma and urine. The glycated haemoglobin (HbA1c), plasma insulin, parathyroid hormone (PTH), calcitonin, vitamin D and 1,25-dihydroxyvitamin D3 concentrations were measured using separate specific ELISA kits according to the manufacturer’s instructions (Elabscience and Biotechnology, Wuhan, China). Micro-ELISA plates were coated with antibodies as part of the standard experimental protocol in the ELISA kits. The plasma samples were pipetted into the appropriate wells, followed by the immediate addition of the appropriate biotinylated detection antibody (50 µl). The samples were then incubated for 45 minutes at 37°C, after which the unbound components were washed away with the supplied wash buffer. After washing, the wells were filled with 100 µl of Avidin-horseradish peroxidase (HRP), which was incubated at 37°C for 30 minutes. After removing the unattached components with a second wash, the substrate reagent (90 µl) was applied to the wells. This was followed by a 15-minute incubation period at 37°C. Finally, a stop solution (50 µl) was applied to the micro-wells to stop the reaction and allow for appropriate measurements. The optical density at 450 nm was determined using a nano-spectrophotometer (BMG Labtech, Ortenburg, Germany). Glycated haemoglobin, insulin, PTH, calcitonin, vitamin D, and 1,25-dihydroxyvitamin D3 concentrations in the samples were extrapolated from their respective standard curves.

Statistical analysis

The mean ± standard error of the mean (SEM) were used to represent the data. Statistical comparisons were performed with Graph Pad InStat Software (version 5.00, Graph Pad Software, Inc., San Diego, California, USA). The student t test was used to determine statistical differences between two independent groups. Pearson’s correlation test was used to determine the association of calcitropic hormones with HOMA-IR and HbA1c. A value of p < 0.05 was considered statistically significant. A coefficient value between ± 0.70 and ± 1.0 was considered strong.

Results

Glycated haemoglobin

Glycated haemoglobin concentrations were measured in the non-pre-diabetic (NPD) group (n = 6) and pre-diabetic (PD) group (n = 6) after the experimental period. It was evident (Fig. 1) that the concentration of glycated haemoglobin was significantly (p = < 0.0001) higher in the PD group as compared to the NPD.
Fasting Plasma Glucose, Insulin And HOMA-IR

Fasting plasma glucose concentrations, insulin concentrations and HOMA-IR were measured in the non-pre-diabetic (NPD) group (n = 6) and pre-diabetic (PD) group (n = 6) after the experimental period. It was evident (Table 1) that the concentrations of fasting glucose (p = < 0.0001) and insulin (p = < 0.0001) in plasma were significantly higher in the PD group as compared to the NPD. Furthermore, the PD group had significantly (p = < 0.0001) higher HOMA-IR value compared to the NPD group, which was in the range of significant insulin resistance (> 2.9), whereas the NPD group HOMA-IR value was within the insulin-sensitive range (1.0).

<table>
<thead>
<tr>
<th>Groups (n = 6)</th>
<th>Plasma glucose (mmol/L)</th>
<th>Plasma insulin (ng/ml)</th>
<th>HOMA-IR values</th>
</tr>
</thead>
<tbody>
<tr>
<td>NPD</td>
<td>4.40 ± 0.20</td>
<td>3.47 ± 0.12</td>
<td>0.68 ± 0.05</td>
</tr>
<tr>
<td>PD</td>
<td>6.72 ± 0.12****</td>
<td>10.87 ± 0.06****</td>
<td>3.24 ± 0.06****</td>
</tr>
</tbody>
</table>

Plasma and urinary calcium from 24-hour urine samples

Plasma and urinary calcium concentrations were measured in the non-pre-diabetic (NPD) group (n = 6) and pre-diabetic (PD) group (n = 6) after the experimental period. It was evident (Fig. 2) that the concentration of calcium in plasma was not significantly (p = 0.0959) changed in the PD group as compared to the NPD. The concentration of calcium in urine was significantly (p = < 0.0001) higher in the PD group as compared to the NPD.

Plasma Pth And Calcitonin

Plasma parathyroid hormone (PTH) and calcitonin concentrations were measured in the non-pre-diabetic (NPD) group (n = 6) and pre-diabetic (PD) group (n = 6) after the experimental period. It was evident (Fig. 3) that the concentrations of PTH (p = < 0.0001) and calcitonin (p = < 0.0001) in plasma were significantly higher in the PD group as compared to the NPD.

Plasma Vitamin D And 1, 25-dihydroxyvitamin D3

Plasma vitamin D and 1, 25-dihydroxyvitamin D3 concentrations were measured in the non-pre-diabetic (NPD) group (n = 6) and pre-diabetic (PD) group (n = 6) after the experimental period. It was evident (Fig. 4) that the concentrations of vitamin D (p = 0.0001) and 1, 25-dihydroxyvitamin D3 (p = 0.0311) in plasma were significantly higher in the PD group as compared to the NPD.

Correlation Between Metabolic Parameters And Calciotropic Hormone Levels
Pearson’s correlation analyses were performed between metabolic parameters and calciotropic hormone levels in the non-pre-diabetic (NPD) group (n = 6) and pre-diabetic (PD) group (n = 6) after the experimental period. It was evident (Table 2) that the concentrations of PTH (r = -0.29; p = 0.58), calcitonin (r = -0.70; p = 0.12) and 1,25-dihydroxyvitamin D3 (r = 0.71; p = 0.11) in plasma were not associated with insulin resistance in the PD state. However, the concentrations of PTH (r = 0.82; p = 0.02) and calcitonin (r = 0.95; p = 0.004) in plasma were positively correlated with HbA1c in the PD state. Furthermore, the concentration of 1,25-dihydroxyvitamin D3 in plasma was negatively correlated (r = -0.93; p = 0.007) with HbA1c in the PD state.

Table 2
<table>
<thead>
<tr>
<th>Metabolic Parameters</th>
<th>PTH (pg/ml)</th>
<th>Calcitonin (pmol/l)</th>
<th>1,25-dihydroxyvitamin D3 (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NPD</td>
<td>PD</td>
<td></td>
</tr>
<tr>
<td>HOMA-IR values</td>
<td>r = 0.55</td>
<td>r = -0.29</td>
<td>r = 0.18</td>
</tr>
<tr>
<td></td>
<td>p = 0.26</td>
<td>p = 0.58</td>
<td>p = 0.73</td>
</tr>
<tr>
<td></td>
<td>NPD</td>
<td>PD</td>
<td></td>
</tr>
<tr>
<td>HbA1c (mmol/mol)</td>
<td>r = -0.13</td>
<td>r = 0.82</td>
<td>r = -0.64</td>
</tr>
<tr>
<td></td>
<td>p = 0.81</td>
<td>p = 0.02 *</td>
<td>p = 0.17</td>
</tr>
</tbody>
</table>

Discussion

Calcium homeostasis is regulated by calciotropic hormones which act on the bone, kidney and intestine [6]. Several studies have shown that calcium homeostasis is disturbed in type 2 diabetes mellitus (T2DM), a condition that is often preceded by pre-diabetes [12, 22]. Furthermore, disrupted calcium homeostasis has shown to promote the onset of hyperglycaemia and insulin resistance [23]. Pre-diabetes was induced in rats in our laboratory using a well-established protocol that included the chronic intake of a high-fat high-carbohydrate (HFHC) diet [14, 15]. These studies have shown that complications in T2DM such as cardiovascular disorders, immune dysregulation and renal failure begin in the pre-diabetic state [15, 16]. The changes to calcium homeostasis that are associated with T2DM have been well-characterised; however, there is paucity in information for the pre-diabetic state [24, 25]. Therefore, in this study the effects of diet-induced pre-diabetes on calciotropic hormones were investigated. Furthermore, this study sought to also investigate the association of calciotropic hormones with glycated haemoglobin and insulin resistance in the pre-diabetic state. Pre-diabetes is a metabolic disorder characterised by early insulin resistance and blood glucose levels that are over the homeostatic range [3]. Physiologically, increased glucose concentrations promote insulin release and increase insulin in circulation, allowing peripheral tissue to absorb glucose [2]. This accounts for plasma glucose concentrations been conserved within the homeostatic range in normal glucose tolerant (NGT) individuals [26]. After plasma glucose levels become conserved, plasma insulin levels return towards the homeostatic range [27]. Furthermore, glucose levels are not constantly elevated in NGT individuals to promote increased glycation of haemoglobin and insulin resistance [27]. However, in the pre-diabetic state there is increased glucose concentration, insulin secretion, HbA1c and early insulin resistance by comparison to normal glucose tolerant individuals [2, 15].
During pre-diabetes, plasma insulin fails to stimulate a response in insulin-targeted peripheral tissue such as skeletal muscle [20]. The accumulation of plasma glucose results in enhanced glycation with haemoglobin [28]. As a result, pancreatic β-cells respond by secreting a greater quantity of insulin to overcome the high glucose concentrations resulting in compensatory hyperinsulinemia [28]. In this study, the fasting glucose concentration, plasma insulin concentration, HbA1c and HOMA-IR index were significantly higher in the PD group than the NPD group. These results corroborated with previous findings that have shown elevated plasma glucose, insulin, HOMA-IR and HbA1c in pre-diabetic patients by comparison to normal glucose tolerant individuals [29, 30]. This study further validates that the consumption of the HFHC diet promotes the development of pre-diabetes, as seen by the impaired fasting glucose, insulin, HbA1c and HOMA-IR value in the range of insulin resistance. This suggests that the body's ability to use glucose in insulin-dependent tissues has been disturbed [16]. The excessive intake of dietary fats, which has shown to increase triacylglycerides levels, may have contributed to insulin resistance [31]. The increased exposure of triacylglycerides to insulin-dependent peripheral tissue induces insulin resistance [31]. Consequently, pancreatic beta (β)-cells may have produced more insulin to compensate for the elevated plasma glucose that is found in insulin-resistant tissue [30]. Calcium homeostasis has been found to be disrupted by elevated plasma glucose concentrations and insulin resistance in T2DM [22, 32].

Calcium is needed for a variety of bodily functions, including signal transmission, secretion of hormones and mineralization of bone [22]. Plasma calcium levels are regulated by PTH, calcitriol and 1,25-dihydroxyvitamin D3 also known as calcitriol which act on the bone, kidney and small intestine [6]. When plasma calcium levels fall below normal, the chief cells of the parathyroid gland produce PTH, which stimulates calcitriol synthesis [6, 22]. The parafollicular cells of the thyroid gland produce PTH, which stimulates calcitriol synthesis [6, 22]. The parafollicular cells of the thyroid gland on the other hand, are triggered to release calcitonin when plasma calcium levels rise above normal [6, 22]. Some studies have shown that plasma calcium concentrations are within the homeostatic range in type 2 diabetic patients [33, 34]. These studies have stated that plasma calcium levels do not change significantly due to calcitropic hormones maintaining a constant plasma calcium concentration, despite variations in calcium excretion [33, 34]. In this study, the PD group displayed no significant change to the plasma calcium concentrations by comparison to the NPD group. These results coincided with prior literature that showed no significant change in calcium levels in plasma among T2DM patients [33, 34]. However, these results contradicted other studies that have found either hypocalcaemia or hypercalcaemia among T2DM patients [7, 25]. The possible reason for no significant change to plasma calcium levels in the PD group of this study may have been due to calcitropic hormones compensating for the changes to plasma calcium concentration. The significant alterations to plasma calcium levels in studies on T2DM patients may have been due to the failure of the body to compensate for the changes to plasma calcium levels [32, 35]. In the current study, it was observed that calcitropic hormones had compensated for changes to plasma calcium levels which may have occurred as a result of pre-diabetes.

The concentrations of plasma calcium are kept within the homeostatic range by calcitropic hormones [23]. Calcium levels in the plasma control the secretion of PTH, calcitriol and calcitonin [36]. Parathyroid hormone with aid from calcitriol increase plasma calcium levels by promoting an increase in calcium absorption in intestine, renal calcium reabsorption and bone resorption whereas calcitonin promotes the opposite [13]. Previous research has shown increased concentrations of PTH and calcitonin in plasma of type 2 diabetic individuals [37, 38]. According to studies the increased PTH concentration may have compensated for the increased calcium loss in urine and the increased plasma calcitonin concentration may have protected the body against the adverse effects of PTH oversecretion [13, 39]. The harmful effects associated with elevated PTH secretion include hypercalcaemia and bone loss [23]. In this study, the plasma PTH and calcitonin concentrations in the PD group were significantly higher than the NPD group. These findings validated prior research that has shown elevated levels of PTH and calcitonin in plasma of T2DM patients [11, 40]. In the PD group of this study, the elevated plasma PTH and calcitonin concentrations may have been
ascribed to the coordination between PTH and calcitonin, in order to stabilize plasma calcium levels. Additionally, PTH levels in plasma may have risen to compensate for the increased urine calcium loss. This may lead to secondary hyperparathyroidism which has shown to be associated with impaired glucose tolerance, decreased insulin sensitivity and increased risk of T2DM [41]. Plasma calcitonin concentration may have increased to compensate for the elevated plasma PTH levels in the PD group.

The kidneys, bone and small intestine regulate urinary calcium homeostasis under the influence of PTH and calcitriol [7]. Nearly 98% of filtered calcium by the kidneys is reabsorbed back into the bloodstream via the renal tubules, which helps to maintain plasma calcium levels [13]. About 60–70% of filtered calcium is reabsorbed in the kidney's proximal tubule [13]. The remaining calcium is reabsorbed along the ascending limb of Henle and distal convoluted under the influence of PTH [13]. It is estimated that less than 2% of filtered calcium is lost in urine due to renal calcium reabsorption [13]. Type 2 diabetes mellitus promotes renal calcium wastage by altering renal functioning, calcium transport mechanisms and damaging renal tubular cells [7, 32]. Furthermore, compensatory hyperinsulinemia as a consequence of insulin resistance has shown to inhibit renal calcium reabsorption in T2DM patients [42, 43]. In this study, the urinary calcium levels in the PD group were significantly higher by comparison to NPD. Furthermore, the urinary calcium levels in the PD group were above the homeostatic range of 15 mmol/L-20 mmol/L, indicative of hypercalciuria [44]. These findings coincide with recent studies that have shown increased urine calcium levels in T2DM patients [45, 46]. Renal impairment is present in the pre-diabetic state, according to studies conducted in our laboratory [16, 19]. The elevated urinary calcium concentration in the PD group may have been due to impaired renal regulatory function induced by pre-diabetes. Furthermore, high glucose levels in the PD state may have directly damaged mechanisms of calcium transport in the renal tubule [47]. In addition, studies in human and rodent models have shown that increased urinary calcium levels were associated with elevated plasma insulin levels [7, 10]. Compensatory hyperinsulinemia as a consequence of insulin resistance has shown to inhibit renal calcium reabsorption [10]. Hyperinsulinemia-induced by pre-diabetes may have promoted increased urinary calcium loss by inhibiting renal calcium reabsorption. Additionally, increased renal calcium load may have also contributed to the higher concentrations of calcium in the urine of the PD group. Diets that have content high in protein have been demonstrated to cause metabolic acidosis, which is manifested by the elevated loss of calcium in urine [48]. The increased renal acid load contributes to bone breakdown and consequently promote hypercalcaemia [48]. Therefore, the kidneys may try to compensate for the increased plasma calcium by excreting it into urine.

Vitamin D is a fat-soluble vitamin that plays a significant role in regulating whole body calcium homeostasis within the endocrine system [49]. Vitamin D is the precursor molecule needed for calcitriol synthesis [49]. Calcitriol is vitamin D in its hormonally active form responsible for increasing plasma calcium concentrations [49]. Previous studies have found reduced plasma vitamin D and calcitriol levels among T2DM individuals [49, 50]. These studies have shown that impaired renal reabsorption of vitamin D binding proteins, impaired intestinal vitamin D absorption and increased sequestration of vitamin D by adipose tissue may be responsible for decreased plasma vitamin D and calcitriol concentrations in T2DM [39, 43]. Furthermore, studies have shown that calcitriol production is impaired due to renal damage, low plasma PTH levels and PTH resistance in T2DM [47, 51]. In this study, the PD group had significantly higher plasma vitamin D and calcitriol levels by comparison to the NPD group. These results contrasted previous findings which have shown decreased plasma vitamin D and calcitriol in T2DM individuals [49, 50]. The PD group in this study was fed a diet high in saturated fats and carbohydrate content by comparison to the standard diet. Diets that contain high fat content have shown to stimulate bile secretion and consequently enhanced intestinal vitamin D absorption [52, 53]. It is plausible that the increased dietary fat content in the HFHC diet may have promoted an increase in vitamin D absorption, accounting for the higher plasma vitamin D levels in the PD group. In addition, the increased calcitriol levels in the PD group may have been a compensatory response to reduced plasma calcium levels
and elevated plasma PTH levels. Elevated plasma PTH levels induce an increase in renal-1 alpha hydroxylase expression [54]. Renal-1 alpha hydroxylase catalyses the conversion of calcifediol to calcitriol, promoting an increase in plasma calcitriol levels as seen in this study [54].

Previous studies have shown that calcitropic hormones participate in the regulation of glucose homeostasis by modulating effects on gluconeogenesis, glycogenolysis and insulin-signaling [23, 39]. Studies have shown that plasma PTH and calcitonin levels were positively correlated with hyperglycaemia and insulin resistance in T2DM [40, 47]. Conversely, previous studies have shown that calcitriol levels in plasma were inversely correlated with hyperglycemia and insulin resistance [12, 40]. Elevated PTH and calcitonin levels were found to promote insulin resistance and hyperglycemia by disrupting the insulin signaling pathway, increasing hepatic glycogenolysis and gluconeogenesis [40]. In contrast, calcitriol has been shown to improve the sensitivity to insulin and tolerance of glucose [8]. In this study, plasma PTH and calcitonin levels were positively correlated with HbA1c but not with insulin resistance in the PD state. Furthermore, plasma calcitriol levels were negatively correlated with HbA1c in the PD group. These results corroborated with previous findings that reported that plasma PTH, calcitonin and calcitriol were associated with HbA1c [39, 40]. However, the lack of association between plasma PTH, calcitonin and calcitriol with insulin resistance contrasted previous studies that have reported associations between these hormones with insulin resistance [23, 32]. These observations may suggest that the elevated plasma PTH and calcitonin levels due to disrupted calcium homeostasis may contribute to the elevated HbA1c concentrations in the PD state. Elevated plasma PTH and calcitonin levels from disrupted calcium homeostasis may stimulate gluconeogenesis and glycogenolysis [55]. It may be speculated that early insulin resistance and beta dysfunction in the PD state may create an unfavourable environment, where increased glucose production stimulated by elevated PTH and calcitonin levels may not be compensated for in a pre-diabetic individual. This may lead to a vicious cycle where disrupted calcium homeostasis in the PD state may promote the development of hyperglycaemia in T2DM. The significant negative correlation between plasma calcitriol and HbA1c in the PD state may suggest a protective role of calcitriol against intermediate hyperglycaemia in the PD state.

The findings of this study may serve of clinical importance in the early detection of type 2 diabetes mellitus. These results may increase our understanding of calcium homeostasis and may provide a possible insight into potential target sites in the treatment of overt T2DM and its associated complications. Furthermore, these findings have elucidated the mechanism of calcium homeostasis in a pre-diabetic rat model. Due to the similarity in the genetic variability of Sprague Dawley rats and humans, these findings may provide an understanding of calcium homeostasis in pre-diabetic humans. Since the current study indicated that some of the complications associated in T2DM such as hyperparathyroidism and hypercalciuria begins in the pre-diabetic state, it is worth translating this research to human studies as a future prospective.

It was evident that hypercalciuria is present in the PD state, an indication of disturbed calcium homeostasis. In addition, plasma calcium levels may have been conserved due to elevated plasma PTH, calcitonin, vitamin D and calcitriol levels in the PD state. Furthermore, plasma PTH and calcitonin levels were positively correlated with HbA1c but not with insulin resistance in the PD state. In addition, plasma calcitriol levels were negatively correlated with HbA1c in the PD state. Although calcitropic hormones try to maintain calcium homeostasis in pre-diabetes, elevated levels of PTH and calcitonin may promote the development of hyperglycaemia in T2DM.

**Conclusion**

Taken together, the effects associated with diet-induced pre-diabetes on calcitropic hormones include elevated plasma PTH, calcitonin, calcitriol and vitamin D concentrations. This was accompanied by elevated urine calcium and
unchanged plasma calcium levels. Collectively, these observations may suggest that calcium homeostasis is disturbed in the PD state but calcitropic hormones compensate for the changes to plasma calcium levels. Furthermore, altered levels of calcitropic hormones in the PD state may contribute to the development of hyperglycaemia and not insulin resistance in T2DM.

**Future recommendations**

In future, the exact mechanisms in which calcitropic hormones contribute to the development of hyperglycaemia needs to be investigated in the PD state.

**Declarations**

**Acknowledgements**

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**Competing interests**

The authors declare that they have no competing interests.

**Ethics approval**

This study was approved by the Animal Research Ethics Committee (AREC) of the University of KwaZulu-Natal, Durban, South Africa (AREC/00003627/2021)

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**Author contributions**

K.N contributed to the study design, conducted the experiments, collected, analysed and interpreted data as well as being involved in writing the manuscript. P.S.N and A.K. was involved in the conceptualization of the study, study design and editing of the manuscript as well as provide funding. All authors have read the manuscript and approved its submission.

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Figures
Figure 1

Concentrations of glycated haemoglobin in the non-pre-diabetic (NPD) and pre-diabetic (PD) groups (n=6 per group). The values are depicted as a mean± SEM. ****= p < 0.0001 when compared to NPD

Figure 2

Concentrations of plasma and urine calcium in the non-pre-diabetic (NPD) group and pre-diabetic (PD) group (n=6, per group). Values are depicted as mean ± SEM. ****= p < 0.0001 when compared to NPD
Figure 3

Concentrations of plasma PTH and calcitonin in the non-pre-diabetic (NPD) group and pre-diabetic (PD) group (n=6, per group). Values are depicted as mean ± SEM. **** = p < 0.0001 when compared to NPD.

Figure 4

Concentrations of plasma vitamin D and 1, 25-dihydroxyvitamin D3 in the non-pre-diabetic (NPD) group and pre-diabetic (PD) group (n=6, per group). Values are depicted as mean ± SEM. * = p < 0.05, *** = p < 0.001 when compared to NPD.