6BBL0320 / 6BBL0321
Project Design in Physiology

APPLICATION FOR A PROJECT GRANT

Details of the applicant:

Surname	Bychkov
Forenames	Ilya
Title	[redacted]
Position	[redacted]

Q2 Name and address of employing institution:

[redacted]

Q3 Type of grant requested:

Project

Q4 Period for which support is sought (state in months):

36 months (3 years)

Q5 Proposed Start Date:

June 2021

Q6 Title of the project (no more than 220 characters):

The role of reactive oxygen species in the development of gestational diabetes mellitus

Q7 SUMMARIES OF PROPOSED RESEARCH (both parts combined should be no more than 400 words).

(a) For scientifically qualified assessors:

Despite increasing incidence of gestational diabetes (GDM) the mechanisms behind the development and resolution of GDM is still not well understood. However, due to the number of similarities of GDM with type 2 diabetes (T2DM), it is likely that the underlying pathophysiology of GDM is similar to that of T2DM. The increased levels of reactive oxygen species (ROS) in the pancreatic β cells have been identified as one of the contributing factors to development of T2DM. This study therefore plans to investigate whether the levels of ROS have a connection to the development of GDM and if antioxidants can be used to reduce the risk or severity of GDM. The study plans to test this in pregnant and non-pregnant C57Bl/6J mice some of which will have diabetes induced using high-fat high sucrose diet. The levels of circulating insulin as well as glucose tolerance will be measure. Furthermore, the pancreases of the mice will be harvested and β cells isolated to carry out further experiments including measurement of β cell mass, DCF and HEt fluorescence, level of apoptosis (using TUNEL staining) and insulin storage and release will be measured in the isolated β cells. Finally, the results from the GDM groups will be compared to those in the control and antioxidant groups to see if the ROS are involved in development of GDM and if antioxidants are effective in treatment or prevention of GDM.

(b) For readers who are not scientifically qualified:

Gestational diabetes (GDM) is a condition similar to type 2 diabetes (T2DM), but it develops in pregnant women during pregnancy. Unlike T2DM, GDM resolves itself after pregnancy. However, despite a large body of research about the T2DM, the mechanisms responsible for GDM are not well understood. However, it is thought that GDM has several similarities to T2DM. For this study, we are planning to investigate if reactive oxygen species (ROS) are involved in the development of GDM. The increase in levels of ROS has been attributed to a wide range of conditions such as heart disease, Parkinson's, and diabetes. This study plans to use pregnant and non-pregnant mice, some of which had GDM induced by feeding them a modified diet. The levels of insulin (a hormone regulating blood sugar) and glucose will be measured in the mice throughout the experiment. The pancreases of the mice will be removed at the end of the study to investigate the changes in the pancreas. In addition, the study aims to investigate whether administration of antioxidants (chemicals that lower levels of ROS) can improve the insulin release and reduce the severity of GDM.

Q8 DETAILS OF RESEARCH PROJECT
Include (a) Aims of the project, (b) Background (c) Experimental design and methods to be used in investigating this problem.

The description of the research project should be no more than 7,000 words long plus diagrams if required (minimum 12pt single spacing). The word count includes all words used, i.e. including words used to refer to the reference list and words that appear in figure legends.

The reference list itself is in the following section and is not included in this word count.

Gestational Diabetes

1. Diabetes Types and Testing

There are 3 types of diabetes seen in the general population as recognised by the 2016 WHO global reports on diabetes. These are type 1 (T1DM), type 2 (T2DM) and gestational (GDM) diabetes mellitus. However, as highlighted in the report, there is no prevalence data for the different types of diabetes. The prevalence data is difficult to obtain due to the lack of routine testing and different diagnostic thresholds used by healthcare providers. However, there was an estimated 422 million people lived with diabetes in 2014 according to the WHO report (World Health Organisation, 2016). While the prevalence of gestational diabetes specifically is unknown, there are estimates from 2015, showing that the GDM affects 14% of world-wide pregnancies or about 18 million births annually.

The main tool used to diagnose patients with GDM is an oral glucose tolerance test. However, as highlighted by Plows et al. in 2018, there were 6 different criteria and regimens widely used in the world prior to 2016. (Ogurtsova *et al.*, 2017) In 2016, the American Diabetes Association and World Health Organisation aligned their GDM diagnostic criteria with the more conservative criteria used by the International Association of Diabetes and Pregnancy Study Group (IADPSG). The IADPSG criteria advise that all pregnant women should undergo an oral glucose tolerance test at 24-28 weeks using a 75g glucose load. (Whiting *et al.*, 2011)

2. Normal Gestational Glucose Homeostasis

During the second and third trimesters, it is normal for women to develop a degree of insulin resistance. The insulin sensitivities of the non-GDM women have been found to reduce between 40% and 80%, depending on the women's BMI, with women with higher BMI showing smaller degree in the sensitivity reduction (Buchanan *et al.*, 1990; Catalano *et al.*, 1991). This was attributed to the women with higher BMI having a lower sensitivity prior to the pregnancy. The increased insulin resistance seen in pregnancy leads the pancreatic β-cells to produce and release an increased amount of insulin in order to maintain blood glucose. This increase in the insulin secretions during the normal non-GDM pregnancies is associated with the enlargement of the islets of Langerhans and hyperplasia of the pancreatic β cells. (Van Assche *et al.*, 1978) The insulin secretion in response to IV glucose has been found to increase significantly in non-GDM pregnant women compared to the same tests carried out after delivery. Interestingly, in the women diagnosed with GDM, the insulin secretion in response to IV glucose remained the same during pregnancy as after delivery. (Bowes *et al.*, 1996) The results obtained by Bowes et al. are summarised in Table 1.

Table 1 Insulin secretions in response to administration of IV glucose. The measurements were taken 20 minutes after glucose was administered. Adapted from (Bowes et al., 1996)

Table 1. Insulin secretions in response to IV glucose (pmol/kg).

	Non-GDM	GDM
During pregnancy	96.2±42.7	65.5±9.3
After delivery	58.3±25.2	57.7±15.7

The exact mechanisms responsible for the insulin resistance seen during normal pregnancy are not fully understood. However, a range of research highlights a number of hormones that potentially lead to the development of the normal insulin resistance during pregnancy. The hormones potentially responsible for this are summarised in Table 2.

Table 2 Effects of hormones on insulin and glucose during normal non-GDM pregnancy.

Hormone	Action on glucose and insulin
Progesterone (Nelson *et al.*, 1994)	Reduced insulin binding Reduced glucose transport Reduced suppression hepatic gluconeogenesis induced by insulin secretion
Cortisol (Giorgino *et al.*, 1993)	Increased insulin resistance Reduced phosphorylation of insulin receptor
Hormone placental lactogen (Nelson *et al.*, 1994)	Reduced insulin secretion Increased insulin synthesis and secretion Increased β-cell mass
Leptin (Virkamäki *et al.*, 1999)	Increased insulin resistance
Glucagon (Del Prato *et al.*, 1987)	Increased insulin resistance

As seen in the table, the majority of the hormones lead to a reduction in the insulin sensitivity in the mother and are released in greater quantities during pregnancy. This phenomenon plays a role in the change of metabolism during pregnancy as the glucose is diverted to be used by the foetus and the mother starts to utilise lipids and protein as the main source of energy during the second and third trimesters. (Di Cianni *et al.*, 2003). Interestingly, the levels of these hormones in both non-GDM and GDM pregnancies are not significantly different. However, the women with GDM have been found to have higher levels of circulating glucagon in the third trimester. As highlighted by Grigorakis et al., the involvement of the glucagon in the pathophysiology of GDM is currently unclear and may just reflect “the relative insulin deficiency” of the women with GDM (Grigorakis *et al.*, 2000). These findings by Grigorakis et al. are further supported by those Okba et al. recently published. Additionally, Okba et al. found that the women with the higher glucagon levels and GDM were at a higher risk of developing type 2 diabetes following the delivery of the baby. (Okba *et al.*, 2020)

The other further complication in the measurement of the prevalence of the GDM in the world is that the majority of the GDM cases resolve spontaneously following birth. This is likely an explanation of the lack of research when comparing to the body of research on the pathophysiology and the effect of the GDM, shown by 20059 results for the “gestational diabetes mellitus” search on the PubMed compared to 133227 results for “type 2 diabetes mellitus” search.

3. Effects of Gestational Diabetes

Recent research suggests that the consequences of the GDM are more complex and long-lasting then thought previously.

Effects on the Mother

One of the major risks of the GDM to the mother is the increased risk of developing type 2 diabetes after delivery of the baby. A study published by Bellamy et al. in 2009 looked at 675000 pregnant women and concluded that the women previously diagnosed with gestational diabetes had “at least a seven-fold increased risk of developing type 2 diabetes mellitus in the future” compared to the women who had normal blood glucose levels throughout the pregnancy. (Kim *et al.*, 2002; Bellamy *et al.*, 2009). However, this risk did not increase throughout the life of the participants. Kim et al. in their meta-analysis showed that the risk was increased in the first 5 years after the delivery, but plateaued after 10 years post-delivery. (Kim *et al.*, 2002)

Furthermore, women diagnosed with gestational diabetes have an increased risk of developing pre-eclampsia (Montoro *et al.*, 2005) and disturbance of the vascular endothelial function (Bo *et al.*, 2007). The combination of these effects places an increased risk of the development of atherosclerotic disease, which is one of the main causes of death in diabetic patients. Furthermore, women diagnosed with GDM have a higher risk of developing diabetic complications such as neuropathy and nephropathy compared to the non-GDM women. (Buchanan *et al.*, 1998; Donnelly *et al.*, 2000)

Effects on the Offspring

However, the major difference of the consequences of the gestational diabetes compared to type 2 diabetes, is that the consequences affect two people: mother and the offspring. Gestational diabetes has been shown to affect the offspring of the mothers who were diagnosed with GDM. The offspring of the women diagnosed with gestational diabetes have been found to have an increased risk of developing obesity, impaired insulin production and sensitivity, and type 2 diabetes (Kaaja & Rönnemaa, 2008).

Most markedly, the offspring exposed to intrauterine high glucose levels in the women with gestational diabetes were eight times more likely to develop type 2 diabetes compared to the control groups. Interestingly, the risk of diabetes in the offspring of type 1 diabetic mothers was only four times greater compared to the control group. This suggests that the offspring of GDM mothers are at a greater risk of developing type 2 diabetes compared to the control and type 1 diabetic groups (Damm *et al.*, 2016).

Furthermore, a study from Gillman et al. has found that the effects on the offsprings' risk of developing obesity were not affected by the treatment of mother's GDM. The study concluded that the treatment of GDM did reduce the weight of the offspring at birth compared to the untreated mothers. However, the BMI of the offspring from the treated group at the age of 5 years was not reduced compared to the untreated groups.(Gillman *et al.*, 2010). However, the Gillman study has only looked at 199 children. It would, therefore, be of benefit to look at a bigger group of children to investigate the effect of GDM treatment on the offspring. Another study found that the GDM treatment of mothers has improved the insulin sensitivity and lowered the fasting glucose of the female offspring. The findings of the Landon study also supported the findings reported by Gillman that the BMI of the GDM offspring were similar irrespective of the treatment regimen of the mothers during pregnancy.(Landon *et al.*, 2015)

The current understanding of the underlying mechanisms of the development of the GDM is largely incomplete. However, the previous research has suggested

that the pathophysiology of gestational diabetes is closely related to that of type 2 diabetes. The similarities between type 2 and gestational have previously suggested that the underlying mechanisms of the two diseases may be similar.(Pendergrass *et al.*, 1995; Zajdenverg & Negrato, 2017; Plows *et al.*, 2018).

Reactive Oxygen Species

The reactive oxygen species (ROS) is a “type of unstable molecule that contains oxygen formed by partial reduction of oxygen" (Ray *et al.*, 2012) and can act as a strong oxidising agent. The examples of reactive oxygen species include superoxide anions (O2−•), hydroxyl radicals (OH•), hydrochlorite ions (ClO-), and hydrogen peroxide(H2O2). The ROS has been linked to the development of a wide range of conditions but are also necessary for the normal function of the cells including cell differentiation (Allen & Balin, 1989), apoptosis, and cell proliferation (Hockenbery *et al.*, 1993).

4. ROS in Type 2 Diabetes Mellitus

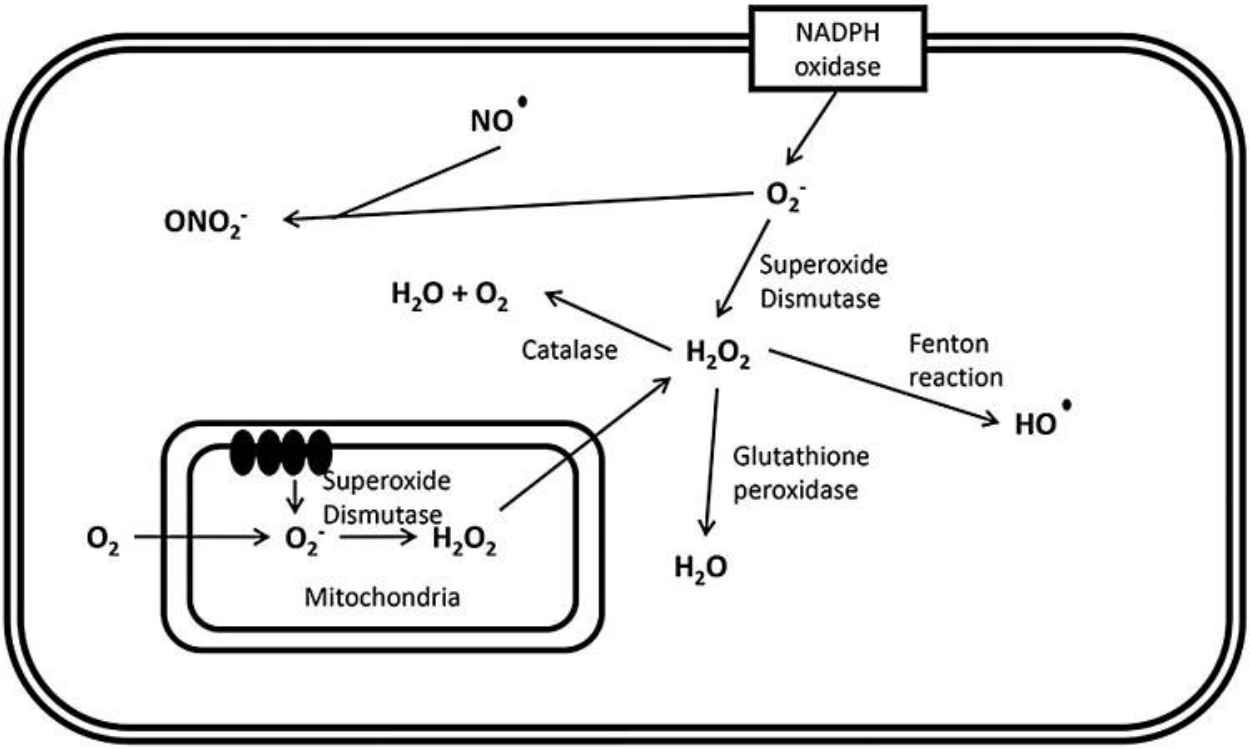

Figure 1 Production of reactive oxygen species within the pancreatic beta cell. Basic mechanisms leading to the production of cellular reactive oxygen species. Adapted from (Gerber & Rutter, 2017)

The ROS has been attributed to the development of type 2 diabetes. However, due to the short half-life of the ROS in the cells and biological fluids, it is extremely hard to single out a specific ROS as the most involved in the pathophysiology of diabetes.(Turrens, 2003; Gerber & Rutter, 2017). Previous research by Newsholme et al., have suggested that the most likely ROS involved in the pathophysiology of the type 2 diabetes is superoxide anions.(Newsholme *et al.*, 2007; Newsholme *et al.*, 2016).

Formation of Superoxide Anions

The superoxide anions are oxygen molecules that have a single unpaired electron and a negative charge. The superoxide anions are produced during the normal functioning of the cells, but superoxide anions can cause a wide range of damage to the cell including DNA damage, lipid peroxidation, amino acid oxidation, and oxidation of protein cofactors. (Barker *et al.*, 2006). The combination of the damage potential and the common production of the superoxide anions has led to the evolution of a range of antioxidant defence mechanisms.

The superoxide anions are most commonly produced by the complex I and complex III of the mitochondrial electron transport chain. Both of these complexes allow a degree of electron leakage as they transfer the electrons to drive the hydrogen ions across the inner mitochondrial membrane. The leaked electrons are then transferred to the oxygen molecules to form the superoxide anions. (Starkov & Fiskum, 2001)

Another producer of the superoxide is the NADPH oxidase enzymes. The NADPH oxidases transfer electrons from the NADPH, that act as the electron donor, to the oxygen molecules. The resulting products of this reaction are NADP+ and superoxide. (Meitzler *et al.*, 2014). The pancreatic β cells have been found to express a range of the NADPH oxidase isoforms. The isoforms found in the pancreatic β cells include NOX-1, NOX-2, NOX-4, NOXA-1, and NOXO-1.

(Oliveira *et al.*, 2003; Shao *et al.*, 2006). The β cells of the type 2 patients have been found to have an increased expression of the NOX-1 isoform, which is known to be a potent producer of the superoxide anions. (Taylor-Fishwick, 2013). Furthermore, the OLETF rats and the db/db mice have been found to have an increased expression of the NADPH oxidases. However, further research is required to identify which isoform of NADPH oxidase is increased. (Nakayama *et al.*, 2005)

Superoxide Anions in Type 2 Diabetes

The conclusion highlighted by Newsholme and Cruzat et al. is based on the increases in the calcium ion concentration seen in the pancreatic β cells due to the influx of glucose at high concentrations. The increased calcium ion concentration in the pancreatic β cells also leads to the activation of the protein kinase C leading to the increased generation of superoxide. (Newsholme *et al.*, 2016) This ROS production induced by the activation of the protein kinase C has been found to be blocked by the addition of diphenylene iodonium, which inhibits NADPH oxidase. This suggests that the NADPH oxidase is one of the proteins responsible for the increased ROS in the pancreatic β cells exposed to high glucose levels. (Morgan *et al.*, 2007). The NADPH oxidase is a protein known to produce superoxide anions, suggesting that superoxide has a role to play in the development of type 2 diabetes. (Gray & Heart, 2010).

However, so far the research of the effects of glucose on the ROS in the islets have produced different results. The currently available research suggests that at low glucose concentrations, the addition of the glucose reduced the levels of ROS in the pancreatic β cells (Gray & Heart, 2010). However, the effects of glucose at concentrations higher than 5 mM has produced opposing results. For example, Martens et al. have found that the increase in glucose concentrations from 5 mM to 20 mM had no effect on the production of superoxide or hydrogen peroxide. Martens et al. suggest that the formation of the NAD(P)H due to the increase in the glucose concentration may act as a suppression mechanism. However, they highlight that further research is currently required to better

understand how this suppression might work (Martens *et al.*, 2005). On the contrary, there are a number of papers that suggest that the increase in the glucose concentrations leads to an increase in the superoxide and hydrogen peroxide formation in the pancreatic β cells. (Bindokas *et al.*, 2003; Leloup *et al.*, 2009)

The ROS have also been found to have a direct effect on the glucose-stimulated insulin release from the isolated pancreatic β cells. However, even these results are not all conclusive. A number of papers suggest that the increase in the ROS concentrations suppresses the release of insulin from the β cells. These papers used different concentrations between 50 uM and 5 mM hydrogen peroxide to stimulate an increase in the ROS. The main difference between the results obtained in these papers was that the papers that used mice isolated pancreatic β cells have found that the increase in ROS have suppressed glucose-stimulated insulin secretion. (Krippeit-Drews *et al.*, 1999; Maechler *et al.*, 1999; Sakai *et al.*, 2003). Furthermore, Sakai et al. had shown that hydrogen peroxide reduced the glucose-stimulated insulin secretion in mice isolated pancreatic β cells in a dose-dependent manner when the hydrogen peroxide concentrations increased from 0 uM to 200uM. Interestingly, all three studies showed that the KCl induced insulin secretion was not affected by the addition of hydrogen peroxide. These findings suggest that the depolarisation of the pancreatic β cells, which leads to the insulin secretion, is not affected by the hydrogen peroxide. However, other studies have found that the increase in ROS has led to an increase in the glucose-stimulated insulin release from isolated pancreatic β cells. However, these studies were carried out using rat pancreatic β cells. These studies also used pharmacological agents to induce production of intracellular and extracellular ROS, rather than directly adding hydrogen peroxide. (MacDonald, 1991a; MacDonald, 1991b; Ebelt *et al.*, 2000). These differences in the experimental design may account for the differences in the results produced in these studies.

Finally, recently published research suggests that the pancreatic β cell mitochondria play a role in the development of diabetes. The study used rotenone, a mitochondrial complex I inhibitor (Heinz *et al.*, 2017), to investigate the role of the ROS produced by the mitochondria during apoptosis of the pancreatic β cells. The study found that the decrease in the ROS production by the rotenone lead to a significant decrease in the apoptosis of the pancreatic β cells. (Wu *et al.*, 2019). These findings suggest that the mitochondrial ROS also play a role in the development of diabetes.

5. Hypothesis and Aims of the Study

Based on the currently available knowledge, this study aims to further investigate the role of the ROS in the development of the gestational diabetes and test the hypothesis described below.

Hypothesis: “Reactive oxygen species produced in the islets of the mice with gestational diabetes play a significant role in the development of gestational diabetes by interfering with the insulin production and reducing the β cell mass in gestational diabetes.”

To answer the above hypothesis, the study has 4 main aims that need to be addressed.

Aim 1: determine the role of reactive oxygen species in the pregnant glucose homeostasis in vivo

Aim 2: Assess the role of reactive oxygen species in the pancreatic beta-cell dysfunction in high-fat diet mice

Aim 3: Assess the effect of reactive oxygen species on isolated pancreatic beta-cell function

Aim 4: Determine the possibility of using antioxidant treatments to prevent or reduce the effects of reactive oxygen species on the pancreatic β cell function in high-fat diet mice.

Experimental Design and Methods

6. Animal Models

To test the hypothesis, the study will be carried out using animal models of gestational diabetes. The two commonly used models of the gestational diabetes are db/+ genetic model and different types of high-fat diet models of gestational diabetes. The other commonly used models such as surgical removal of sections of the pancreas and use of streptozotocin to induce the diabetes have been "discarded" due to their effects on the β cell mass, making them unsuitable for the investigation of the aim 4. (Abdul Aziz *et al.*, 2016)

The db/db mice are commonly used in the research of diabetes. These mice have a mutation in the leptin receptor, which makes these mice more susceptible to the development of obesity, insulin resistance, and type 2 diabetes (Berglund *et al.*, 1978; Arakawa *et al.*, 2001). However, the db/db mice have been found to be sterile and are therefore not used in the research of the development and the effects of the gestational diabetes (Chua *et al.*, 1996; de Luca *et al.*, 2005). However, db/+ mice are fertile and are commonly used in the research of gestational diabetes. The db/+ mice produce sufficient levels of the normal leptin receptors to maintain normal glucose homeostasis when they are not pregnant. However, the changes that occur during pregnancy that affect the glucose homeostasis are sufficient to cause the db/+ mice to develop gestational diabetes. However, these mice do not fully represent the development of gestational diabetes in humans due to the mutations of the leptin receptor, not seen in the human subjects, limiting their utility as a model of the development of the gestational diabetes. (Yao *et al.*, 2015)

The model that the study would like to use is the high-fat diet model using C57BL/6 mice and the high-fat sucrose diet, which is a commonly used combination for the research of the development mechanisms of gestational diabetes. (Rees & Alcolado, 2005; Liang *et al.*, 2010) The high-fat sucrose diet has been previously shown to be effective in the induction of the gestational diabetes without causing distress to the animals during the experiment. (Williams

et al., 2014). The control and high-fat high sucrose diets used are based on the paper by Pennington et al. The high-fat high sucrose diet has 45% fat and 17% sucrose, while the control diet has 17% fat and 2.4% sucrose. These diets have been shown to be effective in the induction of the gestational diabetes by the results from the paper published by Pennington et al. (Pennington *et al.*, 2017). The high-fat high sucrose diet also provides a more accurate representation of the mechanisms of development of gestational diabetes in humans (Williams *et al.*, 2014)

Groups

The study will use 6 groups of animals with 16 animals in each group, meaning a total of 96 animals will be used in the study. This sample size was calculated using the area under the curve data for the blood glucose levels over a glucose tolerance test carried out on the glucose-intolerant mothers. The sample size of 16 would have a 90% chance of detecting a significant difference. This sample size is also similar to those seen in the previous studies, such as that done by Pennington et al. (Abdul Aziz *et al.*, 2016; Pennington *et al.*, 2017).

The six groups will have the treatments outlined in the table below:

Table 3 The breakdown of the test groups with the treatments, n-values, and the aims the group will be used to address.

Group	Treatment	n	Aim
1	Standard diet + non-pregnant control	16	1,2,3
2	Standard diet + pregnant	16	
3	High-fat high sucrose + non-pregnant	16	
4	High-fat high sucrose + pregnant (GDM)	16	
5	High fat high sucrose + pregnant + antioxidant	16	4
6	High fat high sucrose + pregnant + vehicle	16	

The groups 1 and 2 will provide us with the control data to investigate the levels and role of the ROS in the normal β cells both prior and during the pregnancy. Group 3 should provide information about whether the high-fat high sucrose diet

has any effect on the levels of ROS or the function of the β cells in the non-pregnant mice. Group 4 will be the “main” study group as it will allow us to see the effect of gestational diabetes on the levels of the ROS, functioning of the β cells and the mass of the β cells. Group 5 will look into the effect of the antioxidant treatment on the levels of ROS and the functioning of the β cells during pregnancy. Group 6 will act as the vehicle control to rule out that the vehicle used to deliver the antioxidant treatment to the mice affect the severity of GDM.

The final two groups will only be used if the results of the first 4 groups suggest that the ROS levels are increased in the mice that developed gestational diabetes. The antioxidant treatment has previously been suggested to have a beneficial effect on the type 2 diabetes, which suggested to us to investigate whether the antioxidants can be beneficial in the prevention of gestational diabetes. The study would use N-acetyl-L-cysteine (NAC), vitamin C and vitamin E combination as the antioxidant treatment. This treatment has been used by Kaneto et al. to show the efficacy of the NAC/vitC/vitE treatment in protecting the β cells in type 2 diabetes. (Kaneto *et al.*, 1999; Matuszczak *et al.*, 2005). Kaneto has tested all three antioxidant treatments in different combinations. The results obtained by Kaneto, led to them concluding that the NAC/vitC/vitE was the most effective out of the different combinations. These groups should provide further evidence for the role of reactive oxygen species in the development of GDM in mice. Furthermore, the results of group 5 should provide information on whether or not it is possible to prevent the development or reduce severity of the GDM in mice.

7. In vivo Measurements

The main in vivo measurement that will need to be carried out in this study is the measurement of circulating insulin and blood glucose levels. These measurements will need to be carried out on both fasted animals (to establish a baseline) and following an oral glucose tolerance test. The method for the in vivo measurements has been described by Andrikopoulos et al. who have tested a

wide range of glucose tolerance testing in the chow-fed and high-fat sucrose diet mice. Andrikopoulos concluded that the oral administration of 2 g/kg of glucose to mice following 6 hours of fasting had produced the most reliable results. Andrikopoulos has also found that the oral glucose tolerance tests carried out in overnight (18 hours) and 24 hours fasted animals were inconclusive as the plasma glucose and insulin levels were significantly suppressed. (Andrikopoulos *et al.*, 2008) The conclusions of Andrikopoulos' study lead to this study planning to carry out the oral glucose tolerance tests on the mice following 6 hours of fasting.

The method of the glucose tolerance test has been previously described by Lamont et al. The mice are anaesthetised using a general anaesthetic, sodium pentobarbitone, to prevent causing distress to the animals. The animals can then be administered 2 g/kg of glucose into the stomach using a gavage needle. The blood tests can then be obtained from the mice at 0, 15, 30, 60 and 120 minutes from a tail vein. In order to prevent anaemia in the tested mice, the red blood cells will be reinfused into the mice through a cannula following centrifugation of the sample and separation of red blood cells from plasma. The blood samples will then be analysed using a glucometer to measure the glucose levels and commercially available rodent insulin ELISA to measure the levels of insulin. (Lamont *et al.*, 2006; Andrikopoulos *et al.*, 2008).

8. Ex vivo Measurements

The study will require a range of ex vivo measurements to be carried out in order to investigate the hypothesis of the study.

Euthanasia

The mice used in the study will be euthanised using exposure to carbon dioxide, followed by decapitation to confirm euthanasia. The pancreas will then be removed following injection of Hank's buffer with collagenase-V (Carter *et al.*, 2009; Veite-Schmahl *et al.*, 2017). The study is planning to use the carbon dioxide euthanasia, despite suggestions of aversion to carbon dioxide, because repeated exposure to isoflurane has been found to cause similar or higher levels

of aversion in the lab mice. (Boulanger Bertolus *et al.*, 2015). Furthermore, repeated administration of isoflurane has been found to reduce the insulin secretions by the β cells (Desborough *et al.*, 1994)

Cell Culturing

The isolated β cells will then be cultured as described by Carter et al. The RPMI 1640 medium with 11 mM glucose, 100 U/ml penicillin, and 100 ug/ml streptomycin will be used to culture the isolated β cells (Andersson, 1978; Carter *et al.*, 2009). The isolated cells will be allowed to incubate free-floating in the culture medium for 20 hours following isolation to allow for recovery from the collagenase digestion and isolation procedure. The experiments will then be carried out within 3 days of isolation. This time frame is chosen as the islet function can degrade in as little as 4-5 days following isolation, despite the optimal culture medium allowing for as much as a week of viability. (Carter *et al.*, 2009)

Pancreatic β cell mass

During a normal pregnancy, as described earlier, the pancreatic β cell mass increases during the pregnancy in order to meet the increased demands for the insulin release. (Rieck *et al.*, 2009). It is, therefore, crucial to understand whether the reactive oxygen species reduce or prevent the increase in the β cell mass and if the treatment with the antioxidants will have any preventative efficacy.

The mass of the β cells will be measured using the histochemical method described by Montanya and Tellez in 2009 (Montanya & Téllez, 2009). The “negative” staining method will be used as the potentially depleted stores of insulin may prevent the methods such as anti-insulin staining from working. The negative staining uses multiple antibodies such as anti-glucagon and anti-somatostatin, in order to stain the pancreatic α cells. The non-stained endocrine cells are then considered to be pancreatic β cells. This method has been used successfully in a number of publications such as in Biarnes et al (Biarnés *et al.*, 2002).

The mass is measured by measuring the ratio of β cells to other cells. This is done by counting the number of β cells on a section of a slide and dividing this by the total number of cells in the section. This will then be repeated for 10 slides for each mouse, and the average will be used for the further calculations. The ratio of β cell: total cell count will then be used to find the ratio of the β cell mass compared to the whole pancreas mass.

Apoptosis of pancreatic β cells (TUNEL)

Apoptosis, also known as the programmed cell death, of the pancreatic β cells has been identified as one of the cellular mechanisms involved in the development of type 1 and 2 diabetes. Apoptosis is characterised by the cell shrinkage, chromatic condensation, DNA fragmentation, and the formation of apoptotic bodies (Kerr *et al.*, 1972). The levels of the apoptosis are of particular interest to this study because the increased levels of reactive oxygen (and nitrogen) species can lead to the activation of the apoptotic pathways due to the antioxidant defences being overwhelmed. (Tomita, 2016) The increase in the levels of the apoptotic makers would suggest an explanation of the β cell mass decrease and provide the potential causative link for the role of the reactive oxygen species in the development of gestational diabetes.

The apoptosis levels will be measured using commercially available terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) stain. The TUNEL stain labels the 3’ terminal of the double-stranded DNA breaks that appear during the late stages of apoptosis. The slides used for the β cell mass experiments will be used for this experiment to reduce the number of animals that must be used for the study. The number of TUNEL positive cells which are also insulin-positive will be counted to measure the ratio of apoptotic to non-apoptotic cells.

Reactive oxygen species levels in pancreatic β cells

The levels of the reactive oxygen species measurements are complicated due to the extremely short half-life and absence of effective markers for the reactive oxygen species directly.

HEt Method

The study is planning to utilise the DCF fluorescence and hydroethidine staining in order to measure the levels of reactive oxygen species in the pancreatic β cells. Both of these markers are commonly used to measure the levels of hydrogen peroxide (H2O2) and superoxide anions (O2-), respectively.

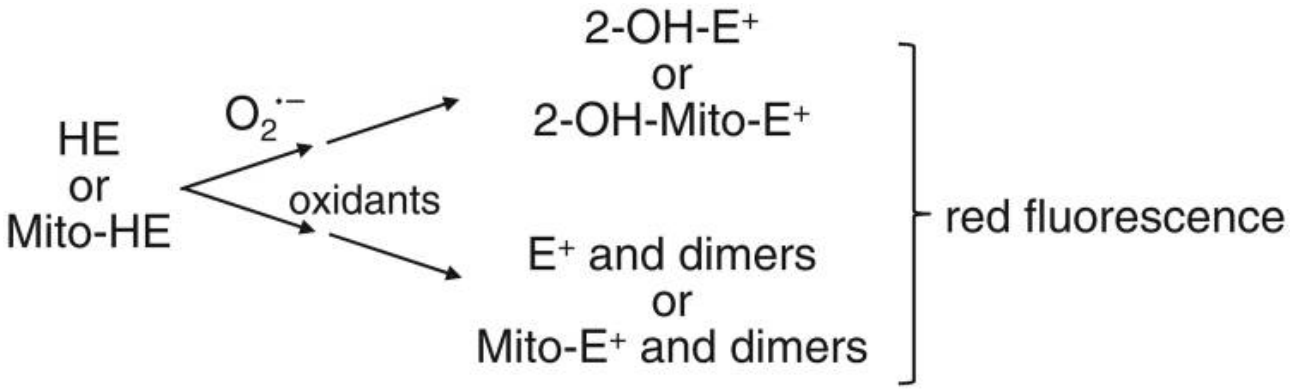

Figure 2 Oxidative reactions of hydroethidine (HEt) and Mito-X. Adapted from (Kalyanaraman et al., 2012)

The hydroethidine (HEt) is a blue fluorescent compound. HEt is cell-permeable which allows in to enter the cell through the plasma membrane and react with the superoxide anions in the cell. HEt has been found to resist self-oxidation. The oxidation products of the hydroethidine are shown in Figure 2. Following oxidation by the superoxide anions, HEt is converted to 2-hydroxyethidium (Bindokas *et al.*, 1996). However, in addition to the conversion to 2-hydroxyethidium, HEt is also converted to ethidium in the presence of hydroxyl ions and reactive nitrogen species. (Kalyanaraman *et al.*, 2012)

The measurements of the 2-hydroxyethidium levels will be carried out using HPLC-fluorescence assay, as described by Kalyanaraman et al., instead of the more commonly used fluorescent microscopy. This is due to the fluorescent microscopy being limited by the assumption that all of the fluorescence in the probe is derived from the reaction of HEt with superoxide to produce ethidium (Kalyanaraman *et al.*, 2014). As have been found by Zhao et al. the HEt produces 2-hydroxyethidium, which produces fluorescence in the same red-light fluorescence range as ethidium. (Zhao *et al.*, 2003; Zielonka & Kalyanaraman,

2010). This means that the results of the fluorescence-based methods may be misleading.

DCF Method

The DCF fluorescence uses 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) as the probe for the presence of reactive oxygen and nitrogen species. The DCFH-DA is a non-fluorescent cell-permeable probe which diffuses through the cellular membrane and is converted to 2,7-dichlorodihydrofluorescein (DCFH) inside the cell by the action of esterases. The DCFH is then oxidised to a fluorescent marker, dichlorofluorescein (DCF). The percentage fluorescence increase can then be used as a measure of the overall oxidative stress in the cell. (LeBel *et al.*, 1992; Gomes *et al.*, 2005)

DCF is no longer used as the probe for the hydrogen peroxide as the DCFH can be oxidised by a wide range of oxidising agents. However, it is widely used as the marker for the overall oxidative stress as the fluorescence increase has been found to be proportional to the oxidative stress. (Wang & Joseph, 1999)

The DCFH-DA experiments will be carried out using a similar method to majority of ROS papers, which was first described by Wang and Joseph. The β cells will be loaded into 96-well plates and allowed to stabilise for 24 hours. The cells will then be incubated with 100uM of DCFH-DA for 30 minutes. Following the incubation period, the DCFH-DA will be removed, and the fluorescence measured using a plate reader using 485nm excitation filter and 530nm emission filter at time 0 min and 30 min. The percentage change in the fluorescence will then be calculated using $[(Ft_{30} - Ft_0)/Ft_0 * 100]$ (where Ft_{30} is the fluorescence at 30 minutes and Ft_0 is the fluorescence at 0 minutes. (Wang & Joseph, 1999; Gujral *et al.*, 2002)

HEt and DCF Limitations

Furthermore, we are aware that the results obtained using DCF and HEt probes cannot be viewed as absolute measurements of the reactive oxygen species as pointed out by Zielonka et al. and Kalyanaraman et al. Both of these probes have

a number of limitations due to the complex interactions with the reactive species found in the cells. However, DCFH-DA and HEt are the most accurate probes currently available and are a commonly used method to measure differences in the levels of reactive oxygen species in the cells. (Zielonka & Kalyanaraman, 2010; Kalyanaraman *et al.*, 2012)

Insulin release from the isolated pancreatic β cells

In order to better understand if the changes in the reactive oxygen species level in the pancreatic β cells affect the insulin secretions, the insulin secretion from the isolated pancreatic beta cells will be measured. The cultured pancreatic β cells will be placed in 12-well plates with RPMI 1640 medium and a range of glucose concentrations. The basal level of insulin secretion will be measured using 3mM glucose. The glucose-stimulated insulin release tests will then be carried out using glucose concentrations of 4, 8, 12, 16, 20mM glucose. The cells will be incubated in the plates for an hour. Following this, the media will be centrifuged to remove the supernatant, and the insulin levels of the supernatant measured using insulin ELISA. (Carter *et al.*, 2009; Nolan & O'Dowd, 2009)

9. Statistical Analysis

The data will be analysed using SigmaPlot 14 software. The data will be presented as mean ± SEM. The analysis of the data will be carried out using ANOVA tests with Tukey's post-hoc test to compare the data from the groups. Differences with the p value lower than 0.05 will be considered significantly different.

Predicted Outcomes and Potential Further Study

10. Predicted Outcomes

The results for all of the experiments carried out with group 1 and 2 should remain unchanged throughout, with the reactive oxygen species and apoptosis levels remaining the lowest of all groups. Group 2 is expected to have a higher level of both circulating insulin and augmented isolated β cell response to glucose. These predictions are based on normal glucose metabolism for the non-

pregnant and pregnant mice. The groups 3, 4, 6 are expected to develop glucose intolerance or diabetes due to the high-fat high sucrose diet feeding. Group 3 is predicted to at least develop mild insulin resistance and thus increased levels of insulin. However, they can also be expected to show slightly higher levels of ROS and apoptosis compared to groups 1 and 2. Group 4 is expected to develop full gestational diabetes and exhibit the highest levels of ROS, apoptosis and β cell mass reduction. Furthermore, the function of the remaining β cells is expected to be significantly reduced.

If the results of the first 4 groups suggest that the ROS are involved in the development of gestational diabetes, the experiments on groups 5 and 6 will commence. This is done in order to reduce the number of animals used for the study if the results of the first stages suggest that the hypothesis will not be supported by the evidence. Group 5 is expected to have the lowest levels of ROS and apoptosis (potentially lower than groups 1 and 2 (Kaneto *et al.*, 1999)), as well as showing improved response to glucose tests compared to group 4. Finally, group 6 is expected to show same results as group 4 as the vehicles used to deliver the antioxidant treatments are not expected to have any effect on the oxidative stress.

11. Further Studies

The further studies of the role of reactive oxygen species would need to be carried out if the evidence obtained in this study supports the hypothesis. The main two pathways of future studies would concentrate on 1) the identification of a specific oxygen species responsible for the development of gestational diabetes and 2) the investigation of the potential causes of the increase in the oxidative stress. The first pathway would currently require a complex range of experiments due to the lack of specific reactive oxygen and nitrogen species markers. The second pathway will likely involve an investigation of the interaction between placenta, foetus, and pancreatic β cells in order to further understand the relationship between oxidative stress and gestational diabetes. Finally, the

identification of clinical markers could be investigated in order to take this research from the laboratory bench to the patients.

Q9 REFERENCES
(Research Project)

Please give citations in full, including the title of paper and all authors using any standard conventional reference format.

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Q10. RESEARCH ON HUMAN PARTICIPANTS OR HUMAN TISSUE:

(a) Does the project involve the use of human subjects or human tissues?

Write yes or no in box: No

If the answer to the above is YES then please state whether the experiments are likely to raise any ethical objections and provide justification for the work as necessary (200 words max):

(b) Does the project involve the use of human subjects or human tissues outside the U.K?

Write yes or no in box: No

If the answer to the above is YES then please provide details below (200 words max):

(c) Does the project involve the use of human embryos requiring a license from the Human Fertilization and Embryology Authority (HFEA) (refer to www.hfea.gov.uk)?

Write yes or no in box: No

If the answer to the above is YES then please provide details below (200 words max):

(d) Does the project involve the use of gene therapy which requires regulatory approval?

Write yes or no in box: No

If the answer to the above is YES then please provide details below (200 words max):

Q11. RESEARCH USING NHS FACILITIES OR PATIENTS:

(a) In the course of your project do you propose to use facilities within the National Health Service and/or does your research involve patients being cared for by the NHS?

Write yes or no in box: No

(b) Suggest a suitable NHS provider to facilitate this research?

None required

Provide details of the NHS facilities required (200 words max):

None required.

Q12 EXPERIMENTS ON ANIMALS:

(a) Does the project involve the use of animals or animal tissues?

Write yes or no in box: Yes

If yes do your proposals include procedures to be carried out on animals in the UK that require a Home Office License?

Write yes or no in box: Yes

If the answer to Q12(a) is YES then please state whether the experiments are likely to raise any ethical objections and provide justification for the work as necessary (200 words max):

In order to understand the role of ROS in the development of GDM, it is necessary to use animal models to better recreate the pathophysiology of the disease. For this study high-fat high sucrose fed C57BL/6 mice will be used to induce diabetes as well as normal chow-fed. The number of animals required for the study has been calculated using power calculation based on the AUC data to produce a 90% power.
The mice will be housed in small groups of 4-6 mice per cage to reduce stress isolation may cause. The invasive manipulations will be carried out with the mice anesthetised following aseptic requirements. Following recovery from the anaesthetic, the mice will be provided with pain relief to control any post-operative pain.
The mice will be euthanized using the exposure to carbon dioxide as described in the background/method section.

Q13 COMMERCIAL EXPLOITATION:

(a) Will the proposed research use technology, materials or other invention that, as far as you are aware, are subject to any patent or other form of intellectual property protection ?

Write yes or no in box: No

If yes please provide details (200 words max):

(b) Is the proposed research likely to lead to any patentable or commercially exploitable results?

Write yes or no in box: No

If yes please provide details (200 words max):

Q14. REASON FOR SUPPORT REQUESTED

(a) Please justify staff requested providing estimates of total cost (200 words max):

Position	**Annual cost (£)**	**Project cost (£)**
1 Post-doctoral researcher	45,000.00	135,000.00
1 Research technician	31,700.00	95,100.00
Grand Total (£)	**230,100.00**	

(b) Please justify any animal costs requested providing estimates of total cost (200 words max):

Item	**Item/Annual cost (£)**	**Project cost (£)**
90 C57Bl/6J mice	15.36	1,382.00
Delivery	28.21	84.63
Cartons	24.78	74.34
Disposal	1.50	135.00
Maintenance (200 days)	0.32	5,760.00
Grand Total (£)	**7,436.37**	

(c) Please justify materials and consumables requested providing estimates of total cost (200 words max):

Item	**Item/Annual cost (£)**	**Project cost (£)**
10x mouse insulin ELISA kits (96 tests each)	444.00	3,340.00
10x TUNEL Assays (50 tests each)	596.00	5,960.00
Histology kits	700.00	2,100.00
General lab chemicals and consumables (e.g. PPE, pipette tips, chemicals)	1,500.00	4,500.00
100mg Hydroethidine	400.00	400.00
1g DCF-DA	62.00	62.00
100g NAC	150.00	150.00
1L vitamin E	111.00	111.00
1kg vitamin C	58.00	58.00
Grand Total (£)	**16,681.00**	

(d) Please justify equipment requested with estimates of total cost (200 words max):

Item	**Item/Annual cost (£)**	**Project cost (£)**
Glucose monitor and strips	500.00	1,500.00
King's College Equipment Access (HPLC column)	1,000.00	3,000.00
Grand Total (£)	**4,500.00**	

(e) Please justify any other costs (travel support etc.) (200 words max):

Item	**Item/Annual cost (£)**	**Project cost (£)**
Project Home Office License	250.00	750.00
Grand Total (£)	**750.00**	

(f) Please provide the total estimated cost of your project:

Salaries (£)
230,100.00
Animals, Equipment, Consumables and Other (£)
29,367.37
Project Total (£)
259,467.37

Q15 TIMETABLE:
Please provide a timetable plan below indicating anticipated progress of the project and highlighting key points and milestones (plan may be in written or schematic form) (300 words max):

0-18 months:
Start of project. First delivery of the mice for groups 1-4. Initial tests such as weight, blood glucose, blood insulin measured. Mice randomly assigned to groups. Treatments begin according to the group description (diet and impregnation). At around month 5, first set of animals euthanized and ex vivo experiments commence. The euthanizing happens every week with 4 animals in each euthanasia group. At around month 11, all animals are euthanized and data is obtained. 7 months of data analysis and further planning of group 5, 6 experiments carried out to ensure the hypothesis is supported.

18-36 months: Delivery of mice for groups 5, 6. Initial tests such as weight, blood glucose, blood insulin measured. Mice randomly assigned to groups. Treatments begin according to the group description (diet, impregnation, antioxidant treatment). At month 23 first set of animals euthanized, and ex vivo experiments commence. The euthanizing happens every week with 4 animals in each euthanasia group. At around month 26, all animals are euthanized, and data is obtained. The final data analysis stages commence. Writing and publication of paper