Universidade do Minho
Escola de Ciências

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Unraveling the impact of hyperglycemia on early embryonic development

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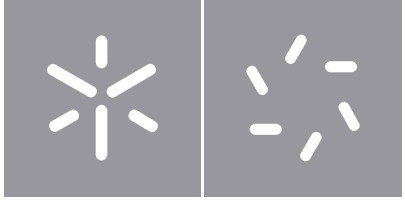

University of Minho
School of Sciences

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Unraveling the impact of hyperglycemia on early embryonic development

Masters Dissertation
Master's in Molecular Genetics

Dissertation supervised by
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Acknowledgments

Once, a good friend of mine told me that, a small positive thought in the morning every day, could change my whole day, week, or month. Now, in this work I would like to acknowledge all the people that stood by my side and helped me to achieve this major goal in my life.

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Statement of Integrity

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

Resumo

Estudo do impacto da hiperglicemia no desenvolvimento embrionário precoce

A diabetes é uma doença crónica caracterizada por níveis anormalmente elevados de glucose no sangue, causando hiperglicemia. A prevalência estimada de diabetes na população adulta está aumentar e aproximadamente um em cada dois adultos com diabetes não é diagnosticado, incluindo mulheres em idade reprodutiva. A hiperglicemia descontrolada contribui para um ambiente prejudicial *in utero* durante a gestação, comprometendo o desenvolvimento embriofetal. A hiperglicemia nas fases iniciais do desenvolvimento embrionário compromete a organogénese e causa anomalias congénita, porém, os mecanismos que as originam ainda não foram totalmente elucidados. Os modelos de mamífero são cruciais para entender os processos responsáveis pelo desenvolvimento de defeitos induzidos pela diabetes, mas enfrentam limitações éticas, práticas ou técnicas. Por outro lado, o modelo do embrião de galinha é adequado para estudar malformações embrionárias porque é acessível para simular distúrbios gestacionais específicos e é semelhante ao embrião de mamífero.

Neste sentido, este projeto teve como objetivo caracterizar o impacto da hiperglicemia nos estadios iniciais do desenvolvimento embrionário usando um modelo *in ovo*. Para a indução de hiperglicemia, ovos de galinha fertilizados foram injetados com diferentes doses de D-Glucose usando, diferentes abordagens, e incubados durante 5 dias. O modelo *in ovo* foi validado através da determinação dos níveis de glucose sanguínea e no ovo. Os embriões foram analisados macroscopicamente para detetar malformações severas. Posteriormente, os tecidos foram analisados a nível molecular.

Os resultados mostraram que é possível induzir diferentes cenários de hiperglicemia *in ovo*, reprodutíveis e independentes do efeito sistémico materno. A administração de glucose causou aumento na taxa de mortalidade e de malformações no embrião, de forma dose-dependente. A análise molecular revelou um aumento nos níveis de expressão de *igf2* e, por outro lado, uma diminuição nos níveis de expressão de *glut1* em fígados hiperglicémicos. Por fim, a atividade da superóxido dismutase, nos embriões malformados, diminui significativamente quando comparados com os controlos.

Este modelo permite de uma forma sistemática, barata e facilmente reprodutível criar diferentes cenários hiperglicémicos durante o desenvolvimento embrionário. Altos níveis de glucose têm um forte efeito teratogénico durante o desenvolvimento do embrião, levando à desregulação do metabolismo da glucose e, alterando o stress oxidativo nas células.

Palavras-chave: Desenvolvimento embrionário; Hiperglicemia; Glicose; Malformações congénitas.

Abstract

Unraveling the impact of hyperglycemia on early embryonic development

Diabetes is a chronic condition associated with abnormally high blood glucose levels causing hyperglycemia. The estimated prevalence of diabetes in the adult population is increasing, and almost one in two adults with diabetes is undiagnosed, including women of reproductive age. Uncontrolled hyperglycemia contributes to a harmful *in utero* environment during gestation, impacting embryofetal development. Hyperglycemia in the early stages of embryonic development increases the risk of impairing organogenesis, which may cause severe developmental abnormalities; however, the mechanisms underlying these events are not yet fully understood. Mammalian animal models are crucial to understanding the pathophysiology of diabetes-induced defects throughout gestation but face ethical, practical, or technical limitations. Conversely, the chicken embryo model is suitable for studying embryo malformations because it is accessible to simulate specific gestational disorders and is similar to the mammalian embryo.

Therefore, this project aimed to characterize the impact of uncontrolled hyperglycemia at the early stages of embryonic development using the *in ovo* approach. For this purpose, fertilized chicken eggs were injected with different doses of D-Glucose through different experimental approaches, and incubated for five days. The *in ovo* model was validated by determining blood glucose levels and glucose levels in the egg environment. Embryos were characterized at the macroscopic level and evaluated for the presence of gross malformations. Subsequently, tissues were analyzed at the molecular level to further support the model.

Results showed that it is possible to induce different scenarios of hyperglycemia *in ovo*, in an extremely reproducible way and totally independent of the maternal systemic effect. The administration of glucose caused an increase in the mortality malformation rate in the developing embryo in a dose-dependent manner. The molecular analysis revealed an increase in *igf2* expression levels and a decrease in the expression levels of *glut1* in hyperglycemic livers. Finally, the activity of superoxide dismutase significantly decreased when compared to control groups.

This model allows a systematic, inexpensive, and easily reproducible way to create different hyperglycemic scenarios during embryonic development. High glucose levels have a strong teratogenic effect during embryo development, disturbing glucose metabolism by the liver and altering the oxidative state of the cells.

Keywords: Embryonic development; Hyperglycemia; Glucose; Congenital Malformations.

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List of abbreviations

AC: after Christ

ANC: anencephaly

BC: before Christ

cDNA: complementary DNA

CDr: Cohen Diabetic resistant rat

CDs: Cohen Diabetic sensitive rat

CNS: central nervous system

CRS: chick ringer's solution

Cve: vehicle control

DAG: diacylglycerol

DIP: diabetes in pregnancy

DM: diabetes mellitus

EC: encephalocele

FIGO: International Federation of Gynecology and Obstetrics

Fw: forward

GDM: gestational diabetes mellitus

GLUT: glucose transporter

HFD: high fat diet

HIP: hyperglycemia in pregnancy

HSD: high-sucrose (72%), and low-copper diet

IDF: International Diabetes Federation

IGF: insulin-like growth factors

IGFBP: insulin-like growth binding protein

LSD: fisher's least significant difference

MC: microcephaly

NaCl: sodium chloride

NADPH: nicotinamide adenine dinucleotide phosphate

OP: optical organ

Pax6: paired box protein 6

PBS: phosphate buffered saline

PCR: polymerase chain reaction

PGDM: pre-gestational diabetes

PKC: protein kinase C

qRT PCR: quantitative real time polymerase chain reaction

RNA: ribonucleic acid

ROS: reactive oxygen species

Rv: reverse

SD: standard deviation

SOD: superoxide dismutase

STZ: Streptozotocin

T1DM: type 1 diabetes mellitus

T2DM: type 2 diabetes mellitus

UNF: unclosed neural folds

WHO: World Health Organization

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Chapter 1. Introduction

1.1. Glucose Metabolism

Glucose is the most abundant monosaccharide, a subcategory of carbohydrates, and it is also one of the most important sugars for organisms since it serves as the major cell energy source, through the process of glycolysis (Niaz et al., 2020).

Blood circulating glucose can be obtained from three sources: intestinal absorption during the fed state, glycogenolysis, and gluconeogenesis. Plants (rice, potato, wheat, etc...) are a major supply source of carbohydrates, like cellulose and starch, in the human diet. Animals lack the enzymes that can break down cellulose, but they have enzymes that can break down starch into smaller glucose molecules (Chandel, 2021). Starch digestion starts in the mouth by the salivary α-amylase and continues in the small intestine by pancreatic α-amylase. Starch digestion is completed in the small intestine, where the two brush border enzymes, isomaltase and glucoamylase, produce glucose which is then transported through the enterocyte into the bloodstream (Ayua et al., 2021). The absorption of monosaccharides leads to an increase in circulating blood glucose levels.

Glycogenolysis is the process of degradation of glycogen, stored in the liver, into glucose. This process is important for maintaining blood glucose levels during meal intervals (Blanco & Blanco, 2017). Finally, after several hours of starvation, gluconeogenesis synthesizes glucose and glycogen from lactate, pyruvate, glycerol, and certain amino acids (Exton, 1972), also contributing to the increasing blood glucose levels.

When blood glucose levels are high, β-cells, in the pancreas, are stimulated to produce insulin. Insulin, then, promotes the translocation of glucose transporter 4, GLUT4, from intracellular storage sites to the plasma membrane of fat and muscle cells (Stöckli et al., 2011). GLUT4 belongs to a family of facilitative transmembrane hexose transporters with 14 members, each of which has a distinct affinity and specificity for sugars, as well as different tissue distributions and physiological function (Khan & Pessin, 2002; Lema-Pérez, 2021; Leto & Saltiel, 2012). In the liver, the primary organ for glucose metabolism, there has been documented the expression of several glucose transporters, like GLUT1, GLUT2, GLUT9 and GLUT10 (Karim et al., 2012).

Once inside the hepatocytes, glucose is phosphorylated into glucose-6-phophate by glucokinase. Glucose-6-phophate is a key molecule in the metabolism of glucose. From this point, three known metabolic pathways can be followed, namely glycolysis, glycogenesis, or pentose monophosphate pathway (Figure 1). The most important pathway of glucose utilization in the liver is glycogen synthesis due to the necessity of generating a fuel reserve that can be used during fasting periods (Adeva-Andany et al., 2016; Burgess, 2015; Hashimoto, 2016). The dysregulation of hepatic glucose metabolism is the main factor for the development of Diabetes Mellitus.

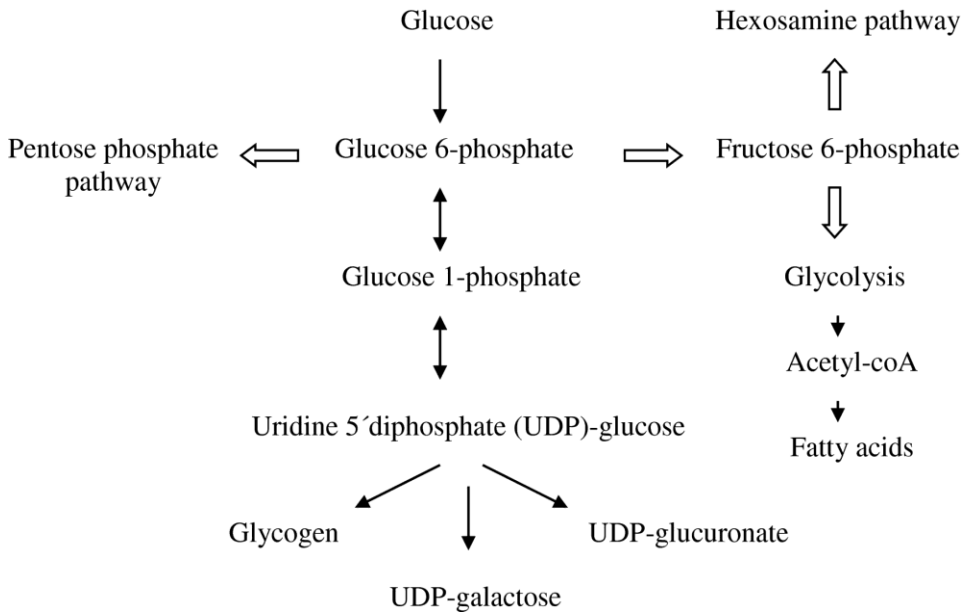


Figure 1: Summary of liver glucose metabolism (Adeva-Andany et al., 2016).

Additionally, the insulin-like growth factors (IGFs) and their binding proteins (IGFBPs) also play an important role in glucose metabolism. Insulin-like growth factor 1 (IGF1) is a liver-derived factor, that can be found in circulation and is mainly responsible for maintaining normal insulin sensitivity, increase glucose uptake, decrease plasma triglycerides, and regulate cholesterol levels (Jensen-Cody & Potthoff, 2021). Insulin-like growth factor 2 (IGF2), mainly synthesized by the liver in adults, inhibits hepatic glucose synthesis and prevents glycogen production (Pouriamehr et al., 2019).

1.2. Introduction to Diabetes

Maintenance of a normal plasma glucose concentration requires a precise balance between glucose utilization, endogenous glucose production and dietary glucose ingestion (Giugliano et al., 2008). Diabetes mellitus (DM) is a group of metabolic diseases characterized by hyperglycemia, technical term

for abnormally high blood glucose levels, in either fasting or after meal states which can result from defects in insulin secretion, insulin action, or both (Alam et al., 2014; American Diabetes, 2009; Kerner & Brückel, 2014).

1.2.1. History of Diabetes

Diabetes has been known since ancient times. Clinical features similar to diabetes mellitus, like excessive thirst and copious urination, were described 3500 years ago by the ancient Egyptians (Ahmed, 2002). Around the 5th century BC, the Indian surgeon Sushruta identified diabetes, by using the term *madhumeha* (honey-like urine) and pointed out not only the sweet taste of the urine but also its sticky feeling to the touch and its ability to attract the ants (Lakhtakia, 2013). However, the term "diabetes" was only introduced by Aretaeus of Cappadocia during the second century AC. Aretaeus gave a precise description of diabetes based only on observation of patients. He described symptoms like increased urine flow, thirst, and weight loss (Bilous et al., 2021; Karamanou et al., 2016).

Historical documents show that Greek, Indian, Arab, Egyptian, and Chinese doctors knew about the condition, but none of them could determine its cause (Bilous et al., 2021). In 1815, Chevreul, a French chemist, proved that the sweetness of diabetic urine was due to glucose. By the end of the 19th century, diabetes was divided into two groups, *diabète maigre* (lean subjects) and *diabète gras* (obese). A few years later, in 1930, diabetes was classified into insulin-sensitive and insulin-insensitive types. These classifications were the forerunners of the etiological classification into type 1 (insulin-dependent) and type 2 (non-insulin-dependent) diabetes (Tattersall, 2017).

1.2.2. Types of Diabetes

Nowadays, World Health Organization distinguishes two main types of DM named type 1 and type 2 diabetes mellitus (World Health, 2019).

Type 1 diabetes mellitus (T1DM) is a chronic autoimmune disease, characterized by absolute insulin deficiency induced hyperglycemia, resulting from the destruction of the pancreatic islet β-cells by the interaction of genetic, environmental, and immunological factors. T1DM accounts for 5 to 10 % of the cases of DM and occurs with increasing incidence in childhood. This type of DM requires an immediate need for exogenous insulin replacement (Bailes, 2002; Katsarou et al., 2017; Paschou et al., 2018).

Type 2 Diabetes Mellitus (T2DM), the most common form of diabetes, usually diagnosed in people above 40 years, is characterized by deficient insulin secretion by pancreatic islet β-cells, tissue insulin resistance and an inadequate compensatory insulin secretory response. T2DM is a heterogeneous disorder, comprising 90 to 95 % of cases in the diabetic syndrome, and is caused by a combination of genetic factors related to impaired insulin secretion or insulin resistance and environmental factors such as obesity, overeating, lack of exercise, stress, and aging (Galicia-Garcia et al., 2020; Ozougwu, 2013).

1.2.3. World Incidence of Diabetes

Over the past years, medical advances have led to a more sophisticated understanding of the causes of diabetes and to an abundance of new tools for managing it. But better treatments have done little to stem the rise of the disease. Diabetes is, nowadays, considered to be in an epidemic state, with 537 million adults with ages between 20–79 years worldwide being diabetic (Figure 2). This represents 10.5% of all adults in this age group (Federation, 2021). This high prevalence of DM seems to be associated with lifestyle changes and globalization, particularly, the sedentary lifestyle of people from First World countries, resulting in obesity, a major cause for the development of T2DM.

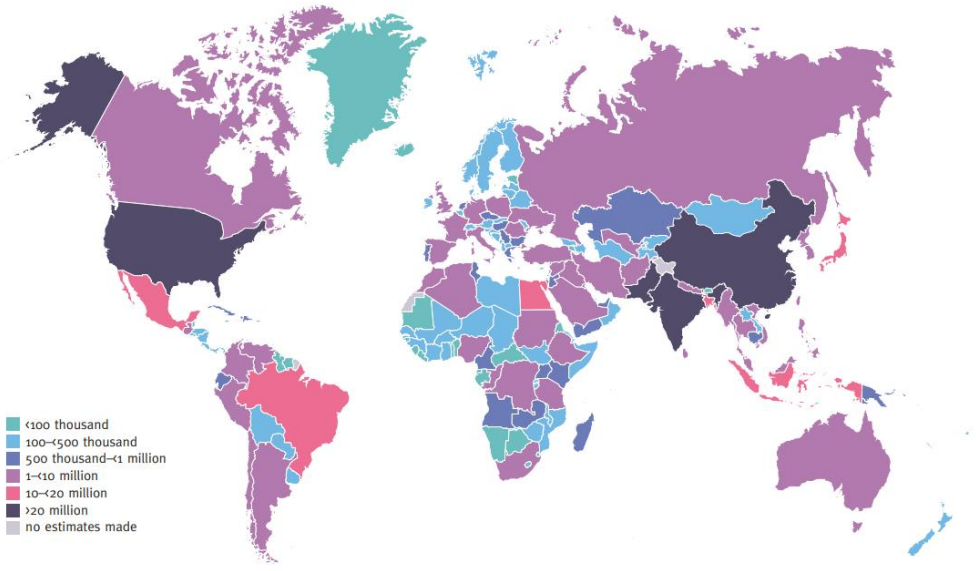


Figure 2: Estimated total number of adults (20–79 years) with diabetes in 2021 (Federation, 2021).

1.3. Pregnancy: a period of adaptations

During pregnancy, a woman undergoes many physiological changes in multiple systems, including cardiovascular, respiratory, and metabolic systems, to maintain a healthy balance between the mother and fetus while ensuring proper fetal development. Glucose and amino acids are the primary nutrients for the developing fetus; in the context of glucose metabolism, these adaptations occur to ensure efficient glucose transport, across the placenta, to the developing embryo while maintaining adequate maternal nutrition (Angueira et al., 2015; Moore, 2018).

1.3.1. Hyperglycemia during pregnancy

According to WHO and the International Federation of Gynecology and Obstetrics (FIGO), hyperglycemia in pregnancy (HIP) can be classified as either pre-gestational diabetes (PGDM), gestational diabetes mellitus (GDM) or diabetes in pregnancy (DIP) (Hod et al., 2015; World Health, 2013). Pre-gestational diabetes, also called preexisting diabetes in pregnancy, includes women with known type 1, type 2 or rarer forms of diabetes before pregnancy (Alexopoulos et al., 2019). Gestational diabetes is defined as glucose intolerance with onset or first recognition during pregnancy. GDM is usually diagnosed in the second or third trimester of pregnancy and usually resolves following delivery (Buchanan et al., 2007; Landon & Gabbe, 2011; Plows et al., 2018). Diabetes in pregnancy is a more serious type of diabetes, which occurs in pregnant women with hyperglycemia that is first diagnosed during the first trimester of pregnancy (< 13 weeks). Unlike GDM, this condition usually persists beyond birth (Guariguata et al., 2014; World Health, 2013). To further distinguish between GDM and DIP, WHO created a glucose level-based criterion (Table 1).

Table 1: Criteria for the determination of diabetes first detected during pregnancy (World Health, 2013).

Gestational diabetes mellitus	**Diabetes in pregnancy**
• fasting plasma glucose - 5.1-6.9 mmol/l (92 -125 mg/dl) • 1-hour plasma glucose - ≥ 10.0 mmol/l (180 mg/dl) following a 75g oral glucose load • 2-hour plasma glucose - 8.5-11.0 mmol/l (153 -199 mg/dl) following a 75g oral glucose load	• fasting plasma glucose - ≥ 7.0 mmol/l (126 mg/ dl) • 2-hour plasma glucose - ≥ 11.1 mmol/l (200 mg/dl) following a 75g oral glucose load • random plasma glucose - ≥ 11.1 mmol/l (200 mg/ dl) in the presence of diabetes symptoms.

The International Diabetes Federation (IDF) estimated that, in 2021, 21.1 million or 16.7% of live births, experienced some form of hyperglycemia during pregnancy. Of these, 80.3% were due to GDM, while 10.6% were the result of diabetes detected prior to pregnancy, and 9.1% due to diabetes (including type 1 and type 2) first detected in pregnancy (Federation, 2021).

1.3.2. Risks of hyperglycemia in pregnancy

Any type of diabetes, if not properly managed, can cause severe risk of obstetric and neonatal complications, morbidity, and mortality. The most common fetal adverse outcome found in pregnancies of women with diabetes are fetal and neonatal loss. Other outcomes that are usually observed are premature delivery (delivery occurring before 37 weeks gestation), fetal growth acceleration and macrosomia (newborn with an excessive birth weight - >4 kg and/or >90th percentile weight).

Early pregnancy (the first 6–7 weeks) is particularly crucial, as this is when organogenesis occurs. During this period, the embryo does not possess a fully developed pancreas, and, as a consequence, there is no embryonic production of insulin. Production and secretion of insulin by the fetus can only be observed at 19 weeks of gestation so, during the first stages of development, the embryo cannot protect itself from hyperglycemic insults.(Holemans et al., 2003; Murphy et al., 2018; Negrato et al., 2012; Parrettini et al., 2020). In case of uncontrolled hyperglycemia during organogenesis, the most common consequences are spontaneous abortion and congenital malformation of the central nervous system, cardiac system, gastrointestinal system, and genitourinary tract (Sugrue & Zera, 2018).

In the present work, the focus will be on diabetes in pregnancy, attempting to characterize the effect of uncontrolled hyperglycemia in the early stages of development of the embryo.

1.4. Animal models to study Diabetes in Pregnancy

The increase in women with DIP led to the necessity to advance the use of experimental diabetic models to gain insight into the molecular basis, the pathogenesis of complications and the utility of therapeutic agents in a multifactorial disease such as Diabetes Mellitus. Experimental models of diabetes and pregnancy need to be chosen according to what aspect of the disease that is being investigated. Animal diabetic models can be obtained by surgical procedures, chemical induction, or the use of spontaneous or genetically derived animal strains (Chatzigeorgiou et al., 2009; Jawerbaum & White, 2010). In the early stages of diabetes research, larger animals were used, like dogs and rabbits.

Later, the scientists preferred to conduct experiments on smaller animals since they are easier to manipulate and involve smaller expenses.

1.4.1. Mammalian animal models

Mammalian animal models, particularly rodent (rats and mice), are widely used to study metabolic disorders. This is due to the fact that because they are mammals, the physiology of mice and rats is closer to that of humans than non-mammalian species (Kleinert et al., 2018). There are several animal models for the study of diabetes; however, in the next subsections, only the mammalian animal models used to study, specifically, diabetes in pregnancy are addressed.

1.4.1.1. Surgical Models

The most invasive procedure is the surgical reduction or removal of the pancreas. This procedure results in the decrease of insulin-producing cells and various other pancreatic cells, which ultimately impair the body's ability to control blood glucose homeostasis.

Although moderate hyperglycemia during pregnancy can be successfully achieved with this procedure, there are strong disadvantages to consider. This major procedure can be challenging in rodent animals because of the particular anatomy of the pancreas and pancreatic ducts. Additionally, the surgery can cause inflammation and other changes in the pancreatic microenvironment not necessarily related to diabetes and, in more severe cases, can lead to pregnancy death, and spontaneous abortion. Finally, the removal of 95% of the pancreas takes up to 3 months until diabetes is settled, and normally, in 90% of the cases, a pancreatic regeneration is observed (He et al., 2020; Kottaisamy et al., 2021; Pasek & Gannon, 2013).

Despite these disadvantages, partial pancreatectomy remains a viable option to study the outcomes of diabetes during pregnancy on maternal health and the subsequent health of the offspring.

1.4.1.2. Chemical models

Various chemicals are currently available to induce diabetes by inducing the death of insulin-producing β-cells or otherwise impairing β-cell function in the experimental animals. Such chemicals are called as diabetogenic agents. Streptozotocin and alloxan are the most commonly used chemical agents to induce diabetes in pregnancy.

Streptozotocin (STZ) is an antibiotic extracted from streptomycin, which has a highly selective toxic effect on the islet β cells of experimental animals, which can make insulin secretion insufficient and increase blood glucose (He et al., 2020). Alloxan acts in two different paths: it selectively inhibits glucose-induced insulin secretion through specific inhibition of glucokinase and causes a state of insulin-dependent diabetes through its ability to induce ROS formation, resulting in the selective necrosis of beta cells (Lenzen, 2008). Depending on the animal strain, dose, route of drug administration, and the life-period in which STZ or Alloxan is administered in rats, severe diabetes or mild diabetes can be generated (Damasceno et al., 2014). Alloxan was the first diabetogenic drug to be established however STZ has almost completely replaced the use of alloxan due to a greater selectivity towards β-cells, a lower mortality rate and a longer or irreversible diabetes induction (Maqbool & Mir, 2019). Despite this, STZ is toxic to organs and tissues other than the pancreatic islet β-cells, so this model does not precisely mimic the human condition. Additionally, to induce T2DM, it is necessary to perform STZ injection alongside with the administration of nicotinamide or combine a high fat diet (HFD) feeding followed by a low-dose multiple STZ injections. Taking all of this in perspective, innumerable factors affect the activity of STZ/alloxan and the extent of diabetes induction. Although it is a basic and commonly used model for inducing diabetes, it is challenging due to problems such as variability, high cost, time, and mortality rates (Akinlade et al., 2021; Furman, 2021; Goyal et al., 2016).

1.4.1.3. Genetic models

The Cohen diabetic rat is the type 2 diabetic model most used during early organogenesis. This model derives from two contrasting strains: the sensitive (CDs) and resistant (CDr). The CDs rats develop type 2 DM when fed a high-sucrose (72%), and low-copper diet (HSD) for 4 weeks, whereas the CDr rats maintain normoglycemia even when fed HSD. This model is a unique rodent model that allows the study of interactions between the genetic background and environmental nutritional factors with the advantage that this is a nonobese model of diabetes, which allows dissociation of the confounding obesity factor from other diabetogenic genes (Ergaz et al., 2012; Ornoy et al., 2009; Ryu et al., 2008). Nevertheless, this model has never been systematically characterized in terms of phenotype or genotype since it was established many years ago, being this a major drawback (Weksler-Zangen et al., 2001).

1.4.1.4. Major difficulties in the use of mammalian models

A range of factors requires consideration when selecting an appropriate animal model to study diabetes. Since the human condition can never be equally simulated in an animal model, caution should be taken to extrapolate the results obtained to the human disease and it is also required to validate the results obtained.

The use of mammalian animal models, like rats and mice, implies various ethical, economic, and experimental issues. In the first instance, to perform medical research in mammalian animal models, it is necessary to have approval from an ethics commission, which needs to evaluate the purposes of the experiment and if it will bring advancement to the knowledge of human physiology. The European Commission, in 2015, stated that investigators should adopt the 3Rs (Replacement, Reduction and Refinement) policy, first developed by (Russell et al., 1959), when working with animals. Replacement comprises the replacement of protected animals for insentient material like cell lines or cultured tissues; mathematical modeling of existing data sets; use of humans, their tissues, or their cells (with permission); or use of immature forms of animals. Reduction means minimizing the number of animals used to obtain valuable and precise information. Refinement involves either reducing the invasiveness of a technique or improving animal welfare and health during scientific studies (Hubrecht & Carter, 2019; Sneddon et al., 2017). After approval, proper care should be taken to provide living conditions for animals. Normally, these animals need special facilities and access to those facilities normally is very restricted (Pasupuleti et al., 2016).

When the subject of the study are pregnant and diabetic animal models, water and food consumption is usually increased, and care should be taken to provide adequate housing considering their increased urination (Jawerbaum & White, 2010).

Another setback to the use of this model is the time necessary to perform one single experiment. Between the induction of hyperglycemia in the mother, the process of mating and the successful pregnancy, several weeks pass by (Gallego et al., 2018). In addition, the economic burden to buy and sustain the facilities and the animal's alimentation and care is too high.

Finally, and having in mind the purpose of this work, mammalian animal models are not the most adequate model since there is no direct access to perform embryo analysis and there are maternal influences in the development.

1.4.2. *Gallus gallus* model

Besides mammalian animal models, other non-mammalian animals, like the avian model, are used in biomedical research. In particular, the chicken embryo is an alternative for studying embryonic development and pathology in a highly detailed and accessible manner (Smith, 1992).

The chicken embryo has several advantages over mammalian models. It is a cost-effective model because there is a high availability of eggs for a low price. It does not require special facilities, since the incubation of the eggs can be performed in an incubator that controls temperature, humidity, and ventilation. In addition, the chicken embryo is a very well characterized model, with a well-established development pattern described in detail by the Hamburger-Hamilton staging system. From this staging system it was possible to observe a similarity between the avian and the mammalian models in the early stages of development (Hamburger & Hamilton, 1992; Rashidi & Sottile, 2009). Moreover, the chicken embryo can be easily accessed from early stages and throughout organogenesis allowing the manipulation of living embryos. Embryos can be removed from the shell for an *ex ovo* culture, or they can be cultured *in ovo*, and, subsequently, their development can be followed for several days (Brown et al., 2003). One important advantage of this model is that until ten days of embryonic life, half of the incubation period, chicken embryos are not considered animals because it's assumed that they are unable to experience pain and for that reason there is no need for special ethics concerns (Aleksandrowicz & Herr, 2015; West et al., 2001).

On the other hand, the chicken embryo also has some disadvantages. The primary setback is that it is not a mammal, so it is sometimes more challenging to extrapolate different studies and applications directly to humans. Additionally, it is difficult to perform mutagenesis screenings and manipulation of the chicken germline genome due to the lack of technologies that could access the embryo before the egg is laid (Dodgson & Romanov, 2004; Vergara & Canto-Soler, 2012).

Despite this, the chick embryo model, has been routinely used as a model for developmental biology, toxicology, cancer research, and immunology. For example, the chicken embryo has been the model of choice for studying the impact of ethanol during pregnancy, a condition that ultimately leads to the development of fetal alcohol syndrome. Tan and co-workers reported that chicken embryos exposed to ethanol 36h post incubation developed severe brain malformations (Tan et al., 2013). Furthermore, other studies demonstrated that alcohol administration to the chicken embryo resulted in an impairment in the cardiac morphogenesis and function (Bruyere & Stith, 1994; Fang et al., 1987). The

chicken embryo has also been used to study the teratogenic effect of thalidomide. It has been established that thalidomide induces limb truncations, and microphthalmia (Knobloch et al., 2007) and eye deformities (Kumar et al., 2016).

Given the difficulty in accessing mammalian embryos, which limits *in vivo* experiments, the chicken embryo model has proven to be an excellent alternative model to study the impact of different substances on embryo development since it is isolated from maternal influence. This feature allows to isolate the specific role in the appearance of malformations (Datar & Bhonde, 2011; Ding et al., 2020; Lawson et al., 2018; Tan et al., 2017; Zhang et al., 2016).

Chapter 2. Objectives

Diabetes is a multifactorial metabolic disorder that has reached epidemic proportions all over the world. The rising incidence of diabetes in pregnancy is overly concerning due to the lack of studies on the specific mechanism by which *in utero* hyperglycemia leads to developmental abnormalities. Because of that, it is crucial to understand the impact of diabetes in pregnancy on the development of the embryo.

The use of mammalian models to study hyperglycemia during pregnancy requires invasive approaches in pregnant animals that implicate tissue damage and elicit ethical considerations. Also, these models do not provide an observation of the real impact of hyperglycemia in the developing embryo, due to the maternal metabolic influence.

In this sense, the main objective of this work is to characterize the impact of uncontrolled hyperglycemia on early embryo development using an *in ovo* chicken model, without invasive interventions.

Thus, this work aimed to achieve the following specific objectives:

- Establish and validate the *in ovo* model for studying the impact of uncontrolled hyperglycemia on early embryo development.
- Determine the impact of uncontrolled hyperglycemia on early embryo development.
- Assess the impact of uncontrolled hyperglycemia on liver metabolic component, specifically, on the expression of key transports and hormones.
- Evaluate the impact of uncontrolled hyperglycemia on oxidative stress, particularly, ROS pathway.

Chapter 3. Materials and Methods

3.1. Ethical statement

The work developed in this project was carried out in the chicken model (*Gallus gallus*) at the early stages of development. The use of fertilized chicken eggs does not require ethical approval from the review board institution or the ethical committee, which is in accordance with the Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. Furthermore, the Portuguese Directive 113/2013 of 7 August 2013 does not contain restrictions on the use of non-mammalian embryos.

3.2. Egg maintenance and incubation, and embryo collection

Fertilized chicken eggs were obtained from a local supplier and stored at 16°C no longer than seven days after laying. Then, the eggs were incubated between 4.5 and 5.5 days in a 49% humidified atmosphere at 37°C.

After incubation, embryos were carefully removed to a Petri dish with PBS 1x and photographed with an Olympus U-LH100HG camera coupled to a stereomicroscope (Olympus SZX16, Japan) in order to register any malformation. Embryos and livers were then dissected under the stereomicroscope and processed for ROS assay and RNA extraction, respectively. In both cases, tissues were snap frozen in liquid nitrogen and stored at -80°C until further use.

3.3. Establishment and validation of a hyperglycemia-induced model *in ovo*

The *in ovo* model can be set up for sustained abnormally high glucose levels, mimicking what occurs when a pregnant woman displays high-glucose levels during the early stages of gestation. The injection of glucose, and other substrates, directly into the egg allows for the observation of the direct effects of hyperglycemia on the embryo without the interference of the mother's metabolism. To validate this model, some parameters after the injection need to be evaluated, specifically, blood and embryo surrounding fluid glucose levels, and the overall aspect of the embryo.

3.3.1. Preparation of Solutions

To establish a hyperglycemia-induced model *in ovo*, several solutions need to be prepared and administered to the egg/embryo. Two solutions used in previous *in ovo* studies as nutrients carriers were used as vehicles: Chick Ringer's solution (Ding et al., 2020; Scott-Drechsel et al., 2013) and NaCl 0.72% saline solution (Cole et al., 2008; Wang et al., 2015).

3.3.1.1. Chick Ringer's Solution

Chick Ringer's solution (CRS) is a special salt solution that is isotonic to physiological pH. In this case, it will be used as a vehicle for other substrates. CRS typically contains sodium chloride (154 mM), potassium chloride (5.6 mM), and calcium chloride (1 mM). It was prepared, filtered through a 0.22 µm vacuum filtration system in a sterile environment, and stored at 4 °C until used.

3.3.1.2. NaCl 0.72%

Another solution used as a vehicle is NaCl 0.72% saline solution. This is a mixture of sodium chloride (salt) and water in 0.72 w/v. The solution was prepared, filtered through a 0.22 µm vacuum filtration system in a sterile environment, autoclaved, and stored at room temperature until used.

3.3.1.3. D-Glucose solution

To induce hyperglycemia, various doses of D-Glucose varying from 0.1 to 1.2 mmol were dissolved in CRS or NaCl 0.72%, filtered through a 0.22 µm syringe filter in a sterile environment, and kept at 4 °C until used. The solutions were stored for a maximum of 5 days, and each aliquot was used once and discarded.

3.3.2. Hyperglycemia induction

Different approaches were used to induce hyperglycemia in the chicken embryo: yolk or air sac injection. The procedures were selected based on previously published works that reported elevated blood glucose levels. In both cases, Chick Ringer's solution and NaCl 0.72% saline solution were used as a control group. This type of control is necessary to confirm that any observed effects are due to the glucose alone and not the solvent.

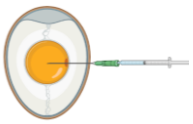
An additional control group, Sham, was performed in all experiments. In this group, no substance was added to exclude the impact of egg manipulation on the embryo's development.

After this first assessment, we opted for the one that gave more consistent results.

3.3.2.1. *In ovo* yolk injection

On day 0 (before incubation), a small hole was made in the middle front shell of the egg with a 21-gauge needle. Before the injection, 900 µL of egg white were extracted with a 24-gauge needle through the same hole to avoid embryo compression inside the egg. Different volumes of CRS (450 µL and 600 µL) were slowly injected directly into the egg yolk. In the case of D-Glucose, the quantity administered ranged from 0.3 mmol to 1.2 mmol (Table 2). The hole was finally sealed with paraffin and eggs were incubated for five days.

Table 2: Summary of the in ovo yolk injection procedure.

Local of Injection	Day	Solution	Quantity (µL)	Quantity (**mmol**)
Egg Yolk 	0	Chick Ringer	450	———
			600	
		D-Glucose	———	0.3
				0.45
				0.6
				0.9
				1.2

3.3.2.2. Air sac injection

3.3.2.2.1. Day 0

For this approach, a 2-cm window was made above the egg air sac before incubation (day 0). Two different methodologies were tested (Table 3).

In the first procedure, with the help of fine forceps, the air sac membrane was carefully peeled off. 250 µL of CRS was pipetted onto the air sac, and in the case of D-Glucose (prepared in CRS), the quantity administered ranged from 0.3 mmol to 0.5 mmol.

For the second procedure, the air sac membrane was preserved intact. 200 µL of CRS and 0.1 mmol to 0.4 mmol of D-Glucose diluted in Chick Ringer Solution were pipetted over the air sac membrane.

In both cases, the window was sealed with medical tape, a material that mimics the eggshell, preventing the accumulation of water due to condensation. Eggs were then incubated for 5 days.

Table 3: Summary of the D0 air sac injection procedure.

Local of Injection	Day	Membrane	Solution	Quantity (µL)	Quantity (**mmol**)
Air Sac 	0	Without	Chick Ringer	250	—
		With		200	
		Without	D-Glucose (CRS)	—	0.375
					0.5
		With		—	0.1
					0.15
					0.2
					0.3
					0.4

3.3.2.2.2. Day 1

Conversely, a 2-cm window was made above the egg air sac one day after incubation (day 1). Similar to the previous method, different approaches were tested (Table 4).

On the first approach, with the help of fine forceps, the air sac membrane was carefully peeled off. 200 µL of CRS was pipetted onto the air sac, and in the case of D-Glucose, the quantity administered was 0.4 mmol.

On the second approach, the air sac membrane was preserved intact. 200 µL of CRS and 0.05 mmol to 0.4 mmol of D-Glucose diluted in Chick Ringer Solution were pipetted over the air sac membrane.

In the third approach, the air sac membrane was preserved intact. 200 µL of NaCl 0.72%, 0.2 mmol, and 0.4 mmol of D-Glucose diluted in NaCl 0.72% were pipetted over the air sac membrane.

In the three scenarios, the window was sealed with medical tape. Eggs were then incubated for four days.

Table 4: Summary of the D1 air sac injection procedure.

Local of Injection	Day	Membrane	Solution	Quantity (µL)	Quantity (**mmol**)
Air Sac 	1	Without	Chick Ringer	200	———
		With	Chick Ringer		
			NaCl 0.72%		
		Without	D-Glucose (CRS)	———	0.4
		With	D-Glucose (CRS)	———	0.05
					0.06
					0.08
					0.1
					0.2
					0.4
			D-Glucose (NaCl 0.72%)	———	0.2
					0.4

3.4. Determination of glucose levels

On developmental day 5 (E5), the medical tape was gently removed to expose the embryo and the fluid surrounding the embryo was collected using a 24-gauge needle attached to a syringe. Next, with the help of a p10 pipette , 10 µL were used to determine glucose concentration.

Live embryos were carefully removed from the egg to a Petri dish. Under the stereomicroscope, and using fine forceps, the amniotic sac was removed, and the embryo was cleaned from residues with a disposable transfer pipette. Then, using a 30-gauge needle attached to a syringe, the aorta was punctured, and blood was pulled out. Subsequently, with the help of a p10 pipette, 10 µL of blood was collected from the syringe to proceed with the measure.

In both cases, glucose concentration was immediately measured with a Contour NEXT glucose monitoring kit (Ascensia Diabetes Care US Inc., Parsippany, USA). The Contour Next blood glucose monitoring kit is a device utilized by individuals with diabetes in home settings to quantify glucose in whole blood. Also, it has already been used for investigation purposes, as reported by (Ding et al.,

2020). This kit has a detection range of 20 to 600 mg/dL and can detect glucose in a minimum sample volume of 0.6 µL.

3.5. Developmental Outcome Assessment

After 5-day incubation, embryos were classified according to their viability into different categories. Embryos that did not exhibit a heartbeat or were non-developed were considered dead. Conversely, the other embryos were macroscopically analyzed, photographed and divided into alive or malformed embryos. A specific score was attributed to the malformed embryos depending on the severity of the malformations. This score is divided into four categories. The first one reflects embryos with non-severe malformations, for example, in the optical organ (OP). The second category includes embryos with two visible malformations or one severe malformation, in this case the criteria was OP and/or encephalocele (EC)/microcephaly (MC)/anencephaly (ANC). The third category comprises three visible or two severe malformations, OP and/or EC/MC/ANC and *ectopia cordis* and/ or cardiac edema. Finally, the fourth category contains embryos with most of the above malformations plus unclosed neural folds (UNF) (Table 5).

Table 5: Detailed score used to categorize malformed embryos

SCORE	MALFORMATION
1	one "not severe" malformation (ex: OP)
2	OP &/or MC, EC, ANC
3	OP &/or MC, EC,ANC & + *Ectopia Cordis* &/ or Cardiac Edema
4	most of the above + UNF (total malformation of the embryo)

3.6. RNA extraction, cDNA synthesis and quantitative real-time PCR

Total RNA was isolated from the collected liver tissue using the TripleXtrator directRNA Kit (GRiSP Research Solutions, Porto, Portugal). Briefly, tissue was mechanically homogenized and total RNA was prepared according to the manufacturer's instructions. RNA (1 µg) was reverse transcribed into cDNA using Xpert cDNA Synthesis Kit (GRiSP Research Solutions, Porto, Portugal).

Primers for GLUT1 and housekeeping gene β-actin were already available in the lab (Fernandes-Silva et al., 2021). Two sets of primers for IGF1 were obtained from (Penha et al., 2011) and (Ji et al., 2021). Likewise, primers for IGF2 were taken from (Liu, Zhi, et al., 2016)(Table 6).

Initially, primers were tested in a conventional PCR (Biorad, USA) with a temperature gradient to determine the best annealing temperature using NZY Taq 2x Green Master Mix (NZYTech, Portugal) and cDNA from livers of control embryos. Even though, two different pairs of primers for the *igf1* gene were tested, neither presented an acceptable expression in liver (Annex 2 - figure 16). However, consistent with (Liu, Zhi, et al., 2016) results, IGF1 expression in embryonic tissues can be found more prominently in muscle so, as a positive control, both pairs of primers for *igf1* gene were tested in heart (Annex 2 - figure 17). Accordingly, due to the poor expression of *igf1* gene in liver, only the expression levels of *igf2* and *glut1* genes were evaluated. Then, primer efficiency was also assessed by performing a cDNA concentration gradient by qRT-PCR (Applied Biosystems 7500 Fast Real-Time PCR System; Applied Biosystems, California, USA).

qRT-PCR was performed using NZY Supreme qPCR Green Master Mix (2x) (ROX; NZYTech, Lisboa, Portugal) according to the manufacturer's instructions and with 1 µl of 1:6 diluted cDNA ($n \geq 6$ per condition). Each sample was run in duplicate. Data were first normalized for β-actin expression levels and expression variations were calculated following the mathematical model 2^(-ΔCt) (Livak & Schmittgen, 2001).

Table 6: Primers and qRT-PCR conditions. Primer sequences forward (Fw) and reverse (Rv), corresponding PCR product size, annealing temperature, and the number of cycles.

Gene	Sequence 5'-3'	Size (bp)	Annealing T (°C)	Cycles
β – actin	Fw – CTTCTAAACCGGACTGTTACCA Rv – AAACAAATAAAGCCATGCCAATCT	100	58	40
glut1	Fw – GCAGTTCGGCTACAACACCG Rv – ATCAGCATGGAGTTACGCCG	222	58	40
***igf1*_P**	Fw –CTTCAGTTCGTATGTGGAGACA Rv – GATTTAGGTGGCTTTATTGGAG	167	58	40
***igf1*_J**	Fw –CCACAAGGGAATAGTGGATGA Rv – CAGAGCGTGCAGATTTAGG	101	60	40
igf2	Fw – AGACCAGTGGGACGAAATAACA Rv – CACGCTCTGACTTGACGGAC	131	58	40

igf1_P - (Penha et al., 2011) and Igf1_J - (Ji et al., 2021)

3.7. Superoxide Dismutase activity assay

Samples were removed from -80°C and, before processing, tissues were washed thoroughly with ice-cold PBS 1x. Next, tissues were homogenized, on ice, with a pellet pestle cordless motor (Kontes Glass, Vineland, New Jersey, USA), in 0.5 mL of PBS with 0.1% of protease inhibitor (Sigma-Aldrich, Missouri, USA) per 100 mg of tissue. Subsequently, tissues underwent sonication (Vibra Cell, SONICS) at an amplitude of 30 for 30 seconds and a pulse of 2. The suspension was centrifuged at 4000 rpm for 10 minutes at 4°C. The supernatant was collected and stored at - 80°C.

SOD activity was measured using the Superoxide Dismutase Colorimetric Activity Kit (Invitrogen, Massachusetts, USA). The supernatants, adequately diluted (1:4), were added to the reaction mixture consisting of Xanthine Oxidase Reagent and the enzyme's substrate. The mixture was incubated at room temperature for 20 minutes, and the absorbance was then measured at 450 nm using a Multimode Microplate Reader Varioskan Flash (Thermo Fisher Scientific Inc, Massachusetts, USA). SOD activity was expressed in units per ml using a Four Parameter Logistic (4PL) Curve (AAT Bioquest, Inc., Sunnyvale, California, USA).

3.8. Statistical analysis

Statistical analysis and the graphical representation were performed using GraphPad Prism version 8 (GraphPad Software, La Jolla, San Diego, USA). One-Way ANOVA was performed and followed by Fisher's Least Significant Difference (LSD) post hoc test for multiple comparisons. All experimental data are presented as mean ± standard deviation (SD). Statistical significance was set for $p \leq 0.05$ (95% confidence level).

D-Glucose is one of the most important biological compounds found in nature, responsible for generating a large portion of the energy potential required for healthy growth and reproduction (Galant et al., 2015). However, in excess, glucose may have a teratogenic effect in the early stages of development.

The first part of this work aimed to establish a hyperglycemic model *in ovo*, to study the impact of hyperglycemia in early embryo development using several methodologies.

4.1. Establishment of the *in ovo* model

4.1.1. The effect of exogenous glucose injection procedure on embryo viability and glucose levels

To determine if the administration of exogenous glucose was effectively causing an increase in blood glucose levels, therefore leading to a hyperglycemic state, features such as embryo viability, blood and egg glucose levels were assessed. This evaluation was necessary to establish the procedure and proceed to further studies.

4.1.1.1. *In ovo* injection

With the *in ovo* injection approach, the mortality rate was very inconsistent. The Sham group presented a survival rate of 79%; we consider that this value accounts for the normal survival rate of E5 embryos. From our experience, some embryos naturally die during development. Control groups (treated with vehicle only, CRS) display higher mortality rates, $Cve_{CRS(300)}$ and $Cve_{CRS(600)}$, than glucose-treated groups. Furthermore, there were random differences in the mortality rate between the glucose-treated groups (Table 7). According to (Scott-Drechsel et al., 2013), yolk-injected embryos, with vehicle solution or glucose, have a higher mortality percentage because the insertion of a syringe by itself into the egg yolk causes a 50% mortality rate for embryos; according to these authors, this procedure is too invasive. Moreover, they describe an increase of 30% in the mortality of D-Glucose embryos compared to the control. In our case, since the values are so inconsistent, we cannot rely on these values.

Table 7: Effect of glucose yolk injection on the survival rate of 5-day chicken embryo.

Experimental Condition	n	%Alive	%Malformed	%Dead
Sham	39	79	8	13
$Cve_{CRS\ (300)}$	41	49	7	44
$Cve_{CRS\ (450)}$	16	75	6	19
$Cve_{CRS\ (600)}$	17	18	12	71
D-Glucose 0.03 mmol	12	83	8	8
D-Glucose 0.3 mmol	23	65	9	26
D-Glucose 0.45 mmol	17	59	24	18
D-Glucose 0.6 mmol	17	41	18	41
D-Glucose 0.9 mmol	10	0	0	100
D-Glucose 1.2 mmol	9	56	0	44

CveCRS(xxx) where xxx is the volume injected in µL

Moreover, blood glucose was measured to confirm if glucose administration induced a hyperglycemic state. In this procedure, Sham embryos presented a value of 87 mg/dL blood circulating glucose. This value can be considered the basal glucose value since no treatment was applied. In the control group, the values ranged from 83 to 96.64 mg/dL. There were no statistically significant differences between the vehicle and Sham controls. These results are in accordance with what was expected because the addition of a saline solution should not alter the glucose values, as demonstrated by (Scott-Drechsel et al., 2013).

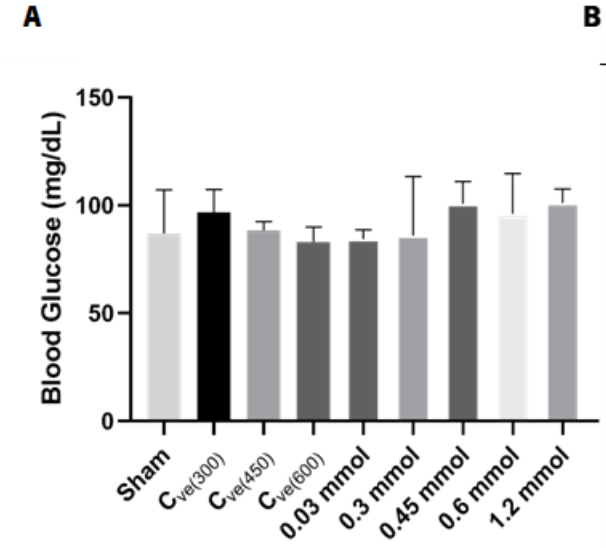
For the control groups, we had to use different volumes of CRS to increase the amount of glucose injected; glucose maximum solubility prevented us from obtaining highly concentrated D-Glucose solutions. Consequently, more volume of a less concentrated solution needed to be administrated to inject a larger amount of D-Glucose. When we compared the different vehicle solutions, we obtained differences between $Cve_{CRS(300)}$ and $Cve_{CRS(600)}$; this result was unexpected because a saline solution should not alter blood glucose levels.

On the other hand, glucose values in the treated groups ranged from 84.40 to 103.70 mg/dL (Figure 3A). The reference study showed that the administration of 0.45 mmol of D-Glucose raised plasma

glucose to 177 mg/dL, a significantly different value compared to the 72 mg/dL of the control embryos (Scott-Drechsel et al., 2013). Our data is not in agreement with the results obtained by these authors since, in our study, the administration of the same amount of glucose only raised the blood glucose to 103.70 mg/dL. When we increased the quantity of glucose administered, the values dropped (87.75 - 101 mg/dL), instead of rising.

Finally, some doses administered revealed a significant difference compared to the controls however these differences were different depending on the volume of CRS administered. Again, this result did not seem reliable since the same dose of glucose should present similar differences with all of the control values, due to the previous mention reason. Also, between doses it was also possible to see significant differences however, due to the high mortality rate, it was not possible to obtain enough measurements to ensure a reliable result (Figure 3B).

Taking into consideration these results, and the lack of consistency between groups, a different approach was conducted.



B

Experimental Condition		*p* value
Sham vs. 0.45 mmol	**	0.0036
Sham vs. 1.2 mmol	*	0.0257
$C_{ve(300)}$ vs. $C_{ve(600)}$	*	0.0253
$C_{ve(300)}$ vs. 0.03 mmol	*	0.0158
$C_{ve(450)}$ vs. 0.45 mmol	***	0.0008
$C_{ve(450)}$ vs. 1.2 mmol	*	0.0166
$C_{ve(600)}$ vs. 0.45 mmol	**	0.0013
$C_{ve(600)}$ vs. 1.2 mmol	**	0.0091
0.03 mmol vs. 0.45 mmol	***	0.0004
0.03 mmol vs. 1.2 mmol	**	0.0057
0.3 mmol vs. 0.45 mmol	*	0.0377
0.45 mmol vs. 0.6 mmol	**	0.0053
0.6 mmol vs. 1.2 mmol	*	0.0343

Figure 3: A- Blood glucose concentration after yolk injection of varying concentrations of D-Glucose at day 0. Results are presented as mean ± SD; B- Statistical analysis of blood glucose assay. *$p < 0.05$; **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. n≥3

For more details, please refer to Annex 1 – table 13

4.1.1.2. Air sac injection

Since the egg yolk injection approach displayed inconsistent results in terms of mortality and blood glucose levels, we decided to employ a different methodology to induce hyperglycemia. We assessed different variations of the same administration method (air sac injection) by changing the day of injection (D0 *vs* D1) and the absence or presence of the eggshell membrane. Different amounts of glucose were evaluated.

4.1.1.2.1. Without membrane

4.1.1.2.1.1. Day 0

With this approach, we obtained around 55% mortality rate for the Sham group and 33% for CRS-treated group. We consider that the Sham mortality rate includes not only the embryos that naturally die during development but also those that have suffered from the experimental procedure. We registered a 70-75% mortality rate for the glucose-treated groups, which is most likely due to glucose itself (Table 8).

Scott-Drechsel and co-workers also performed a similar assay, however the drops of glucose were performed along four consecutive days (from E0 to E3). With this methodology, they reported a 1% of mortality rate for the vehicle control and 10% for the 0.0075 mmol D-Glucose dose (Scott-Drechsel et al., 2013). These results are quite different from ours, which can be due to the differences in the injection protocol, like time points and the glucose dose administered, which was considerably lower than the ones used in our study. Nonetheless, due to the poor control results we did not trust the procedure by itself.

Table 8: Effect glucose air sac injection without eggshell membrane, on day 0 (prior incubation) on the survival and malformation rate of 5-day chicken embryo.

Experimental Condition	n	%Alive	%Malformed	%Dead
Sham	11	27	18	55
$Cve_{CRS\ (250)}$	9	33	33	33
D-Glucose 0.375 mmol	8	13	13	75
D-Glucose 0.5 mmol	10	10	20	70

CveCRS(250) where 250 is the volume injected in µL

To confirm if hyperglycemia was being induced, blood glucose was assessed. The data collected show that there is a significant difference between the controls and the treated embryos (Figure 4) with blood glucose levels rising from 109.50mg/dL in the CRS group to 154 mg/dL in D-Glucose 0.375 mmol treated group and decreasing to 105 mg/dL in D-Glucose 0.5 mmoL treated group.

The previous study that sought to induce hyperglycemia for several days, and used different concentrations of D-Glucose, reported 80 mg/dL of blood glucose in treated embryos which means that they could not induce sustained hyperglycemia in the chicken embryos (Scott-Drechsel et al., 2013). However, with the increase in the amount of glucose administered, we expected to detect a significant increase in blood glucose levels. In fact, with this approach, we were able to increase blood glucose circulating levels in one of the treated groups, however, due to the elevated mortality rate we would need an enormous amount of fertilized eggs conduct the study. Our goal was not to induce embryo mortality but hyperglycemic and viable embryos.

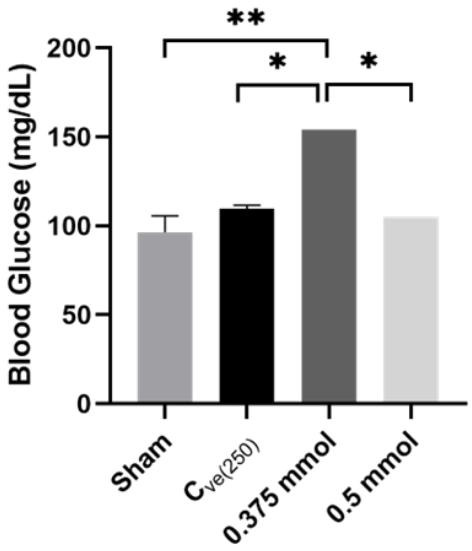


Figure 4: Blood glucose concentration of embryos after air sac injection, without membrane, of varying concentrations of D-Glucose at day 0. Results are presented as mean ± SD. *$p < 0.05$; **$p < 0.01$. n≥1

For more details, please refer to Annex 1 – table14

4.1.1.2.1.2. Day 1

In the very beginning of mammalian development, the embryo is not immediately exposed to the maternal blood. In fact, contact with maternal blood flow only happens after embryo implantation in the

uterus walls, four weeks after conception (Jauniaux et al., 2000). In this sense, we decided to perform a new set of glucose injections after 24 hours of incubation (E1), adapting what was described by (Ding et al., 2020; Tan et al., 2017). In this approach and based on our previous finding we tested only an intermediate glucose dose.

In this case, the mortality rate for the control groups was high, 57% for CRS and 33% for Sham. Moreover, the Sham group only presented malformed and dead embryos (Table 9). This is a clear indicator that the procedure by itself is compromising embryo development. The removal of the eggshell membrane has a high impact on embryo survival and development. So, the 57% of mortality obtained in the vehicle control can be related to the high mortality caused by the procedure.

Although there is no report of an experiment performed with these conditions, the procedure used by (Ding et al., 2020) served as a base for setting this protocol. When we looked at the survival rates presented by these authors, we questioned their survival rate since they obtained 100% of viable embryos in the control groups; we know that some embryos naturally die during development and with such a low number of eggs in the control condition (n=8 for E2 injections) we consider that it is extremely difficult to obtain such results.

Table 9: Effects of 1 day post incubation glucose air sac injection, without membrane, on the percentage of 5-day chicken embryo death and gross abnormality.

Experimental Condition	**n**	**%Alive**	**%Malformed**	**%Dead**
Sham	6	0	67	33
$Cve_{CRS\ (200)}$	7	43	0	57
D-Glucose 0.4 mmol	8	13	50	38

CveCRS(200) where 200 is the volume injected in µL

Due to the poor viability results in this approach, it was impossible to obtain a representative number of blood glucose measurements and determine if there was an increase in blood glucose levels (Figure 5). The value of blood glucose in the control group, 100.50 mg/dL, was slightly lower than the value presented by (Ding et al., 2020), 118.7 mg/dL. Moreover, Ding's group performed the hyperglycemia induction on day 2 post incubation and used different concentrations of D-Glucose. The blood glucose value obtained in the 1g/kg injected at E2 and measured at E6, 159.1 mg/dL, is very different from the value obtained in our study, 106 mg/dL. However, as previously mentioned, we were only able to perform one measurement and this value is not reliable.

For all the above-mentioned reasons, we discarded this procedure and searched for a less invasive technique.

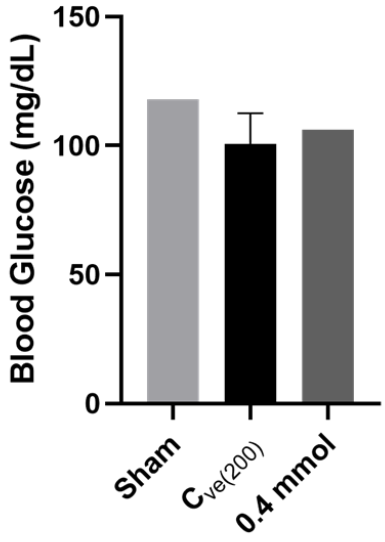


Figure 5: Blood glucose concentration of embryos after air sac injection, without membrane, of 0.4 mmol of D-Glucose at day 1 Results are presented as mean ± SD. n≥1

For more details, please refer to Annex 1 – table 15

4.1.1.2.2. With membrane

4.1.1.2.2.1. Day 0

The previous techniques demonstrated that the procedure was having a negative impact on embryo development. In this sense, we tested a similar approach (air sac injection) but without removing the eggshell membrane (Miller et al., 2005). Although they performed the injections during 3 consecutive days, we opted to perform the injections in a single day to avoid introducing more variables to the study.

With this adjustment, we obtained a low mortality rate associated with the Sham group (16%), which validates the surgical procedure since this percentage represents embryos that naturally die during development. This conclusion is based on our in-house experience; eggs not submitted to procedure normally exhibit a 10-20% mortality rate. The administration of D-Glucose led to an increase in mortality (20%-55%) compared to Sham (16%) and vehicle control (30%). Also, the malformation rate progressively increased in a dose-dependent manner; we recorded the highest value for D-Glucose 0.3 mmol (25%). In the case of D-Glucose 0.4 mmol the malformed rate is lower because the mortality rate increased significantly (Table 10).

(Miller et al., 2005) reported 86% of living embryos for the control group with injections performed in three consecutive days. Similar yet lower values are seen in our experiment. These results can be explained by the fact that the number of eggs analyzed in the present study was much higher (n≥47) than the sample size of the reference study (16≥n≤18) (Miller et al., 2005). Miller and co-workers administered lower amounts of D-Glucose compared to those we tested; nonetheless, it was possible to observe a decrease of around 30% (54.55% for the lowest dose and 51.26% for the highest dose) in the survival rate of glucose administered groups when compared to the control. In our case, there is also a decrease in the survival rate, and in a dose-dependent manner. These results lead us to believe that this procedure was much less invasive and could be a step in the right direction.

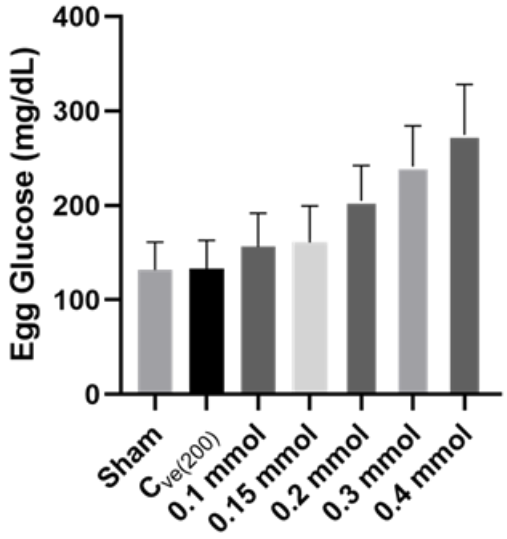
Table 10: Effects of prior incubation glucose air sac injection, with membrane, on the percentage of 5-day chicken embryo death and gross abnormality.

Experimental Condition	**n**	**%Alive**	**%Malformed**	**%Dead**
Sham	106	72	12	16
$Cve_{CRS\ (200)}$	112	65	4	30
D-Glucose 0.1 mmol	93	63	16	20
D-Glucose 0.15 mmol	102	56	21	24
D-Glucose 0.2 mmol	47	26	23	51
D-Glucose 0.3 mmol	55	33	25	42
D-Glucose 0.4 mmol	49	24	20	55

CveCRS(200) where 200 is the volume injected in µL

Given that the previous results showed an improvement in the technique, and to further confirm the induction of hyperglycemia, we measured the levels of glucose in the embryo surrounding environment. The injection of D-Glucose into the egg air sac created a hyperglycemic environment around the embryo. The values of glucose increased significantly from 132.7 mg/dL in the control group to 241 mg/dL in the highest dose of glucose administrated (0.4 mmol) (Figure 6). It was not possible to conduct a comparison of these values with the literature because this measurement was not performed by Miller's group.

A



B

Experimental Condition		*p* value
Sham vs. 0.1 mmol	**	0.0036
Sham vs. 0.15 mmol	***	0.0005
Sham vs. 0.2 mmol	****	<0.0001
Sham vs. 0.3 mmol	****	<0.0001
$C_{ve(200)}$ vs. 0.1 mmol	*	0.0114
$C_{ve(200)}$ vs. 0.15 mmol	**	0.0020
$C_{ve(200)}$ vs. 0.2 mmol	****	<0.0001
$C_{ve(200)}$ vs. 0.3 mmol	****	<0.0001
$C_{ve(200)}$ vs. 0.4 mmol	****	<0.0001
0.1 mmol vs. 0.2 mmol	****	<0.0001
0.1 mmol vs. 0.3 mmol	****	<0.0001
0.1 mmol vs. 0.4 mmol	****	<0.0001
0.15 mmol vs. 0.2 mmol	****	<0.0001
0.15 mmol vs. 0.3 mmol	****	<0.0001
0.15 mmol vs. 0.4 mmol	****	<0.0001
0.2 mmol vs. 0.3 mmol	***	0.0002
0.2 mmol vs. 0.4 mmol	****	<0.0001
0.3 mmol vs. 0.4 mmol	***	0.0006

Figure 6: A- Egg environment glucose concentration of embryos after air sac injection, with membrane, of varying concentrations of D-Glucose at day 0. Results are presented as mean ± SD; B- Statistical analysis of egg environment glucose assay. *$p <$ 0.05; **$p <$ 0.01, ***$p <$ 0.001, ****$p <$ 0.0001. n≥29

For more details, please refer to Annex 1 – table 16

These results prompt us to measure blood glucose levels in all groups (Figure 7). However, despite the hyperglycemic environment, there were no variations in the embryo blood glucose compared to the control group (Cve = 94.71 mg/dL). Our reference study measured glucose levels after 18 days of incubation and detected a significant increase in glucose values. It is well described that blood glucose concentration increases naturally with the development, being 214 mg/dL in newly-hatched chickens (Salmanzadeh et al., 2019). For this reason, we cannot compare the values obtained by E5 with E18. Since we could not provoke an increase in glucose circulating levels, we abandoned this strategy.

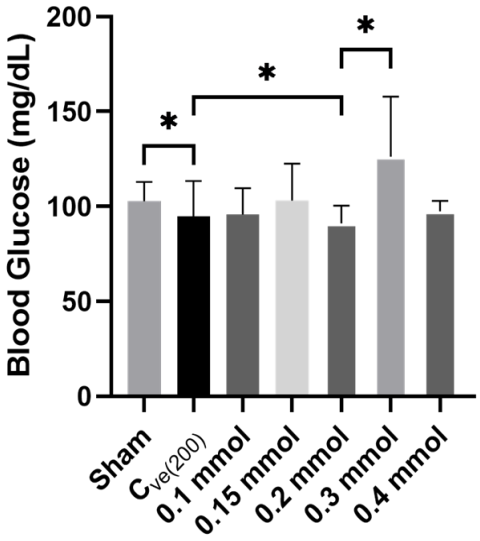


Figure 7: Blood glucose concentration of embryos after air sac injection, with membrane, of varying concentrations of D- glucose at day 0. Results are presented as mean ± SD. *$p <$ 0.05. n≥3

For more details, please refer to Annex 1 – table 17

4.1.1.2.2.2. Day 1

Considering that the embryo is not immediately exposed to the maternal blood at the beginning of development (Jauniaux et al., 2000), we decided to perform a new set of glucose injections after 24 hours of incubation and maintaining the eggshell membrane.

With this methodology, the mortality rate of the Sham (18%) and the control (21%) group was similar. This technique revealed a severe increase in the malformation rate (75%) for the 0.08 and 0.1 mmol of glucose. Moreover, for the highest doses administrated, 0.2 and 0.4 mmol of glucose, the mortality rate increased from 21% in the control group to 57% and 75%, respectively. Unexpectedly, in the doses of 0.08, 0.1, and 0.2 mmol of glucose, there was no live embryo registered (Table 11). Our reference study does not allow us to take conclusions about our results because they did not perform the injections only on day 1 but in three consecutive days.

When compared to our previous results (E0 *vs* E1), the mortality rate from the Sham group did not suffer alterations however the values for the vehicle control slightly decreased. The mortality and malformation rate from the glucose groups drastically increased. In the previous approach, for the 0.1 mmol group, we detected 16% of malformed embryos. This 16% contrast with the 75% of malformed embryos detected with this methodology. Regarding the 0.2 mmol group, despite the abnormal survival rate, the percentage of dead embryos was very similar. Finally, in the 0.4 mmol group, the dead rate

increased 20% when compared to the previous method however the number of embryos analyzed was much smaller.

Despite this, our results were not considered normal due to the lack of live embryos in three glucose doses administered to the embryos.

Table 11: Effects of 1 day post incubation glucose air sac injection, with membrane, on the percentage of embryo death and gross abnormality.

Experimental Condition	n	%Alive	%Malformed	%Dead
Sham	113	71	12	18
$Cve_{CRS\ (200)}$	43	65	14	21
D-Glucose 0.05 mmol	8	38	25	38
D-Glucose 0.06 mmol	7	57	43	0
D-Glucose 0.08 mmol	8	0	75	25
D-Glucose 0.1 mmol	8	0	75	25
D-Glucose 0.2 mmol	7	0	43	57
D-Glucose 0.4 mmol	8	13	13	75

CveCRS(200) where 200 is the volume injected in µL

Even though the viability assessment displayed odd results, we measured glucose in the embryo environment. There were no significant differences between the control group (118.80 mg/dL) and the glucose groups, except for the 0.4 mmol group (266.30 mg/dL) (Figure 8). Additionally, all the doses showed a significant difference compared to the 0.4 mmol dose. Because of the absence of live embryos, we did not measure glucose levels in the 0.2 mmol group. For this reason, it is difficult to understand if the increase in the environment glucose is a dose-dependent characteristic or if it is a random increase justified by the low number of measurements (n=3), which may not be representative enough to compare with the data collected from the other experimental groups. So, this last result cannot be considered trustworthy. We could not find in the literature data to support or oppose our results.

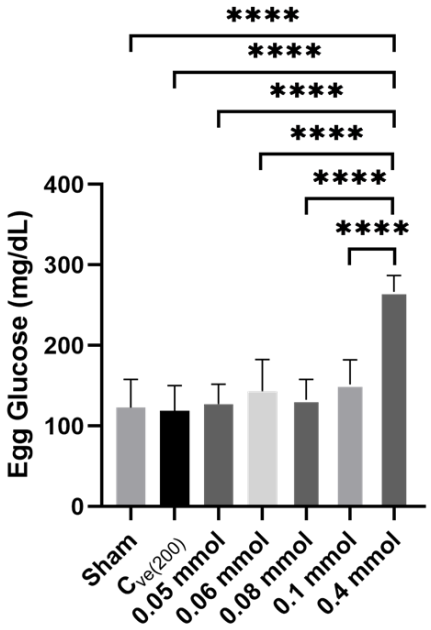


Figure 8: Egg environment glucose concentration of embryos after air sac injection of varying concentrations of D-Glucose at day 1. Results are presented as mean ± SD. ****$p <$ 0.0001. n≥3

For more details, please refer to Annex 1 – table 18

Regarding blood glucose levels, no significant differences were found between the groups. Sham group and vehicle control group presented similar values, 92.40 mg/dL and 97.38 mg/dL, respectively. The values of blood glucose for the glucose treated groups ranged from 88.67 mg/dL to 107 mg/dL (Figure 9). In some groups, it was not possible to obtain a substantial number of measurements due to the higher mortality and malformation rate in the glucose-treated conditions.

Moreover, these results are very similar to the ones obtained in the previous methodology however no significant differences were obtained from the controls to the groups of D-Glucose doses.

To conclude, we were not able to replicate the results obtained by (Ding et al., 2020; Scott-Drechsel et al., 2013) and (Miller et al., 2005). We reproduced the methods described in these studies to the best of our ability but without success.

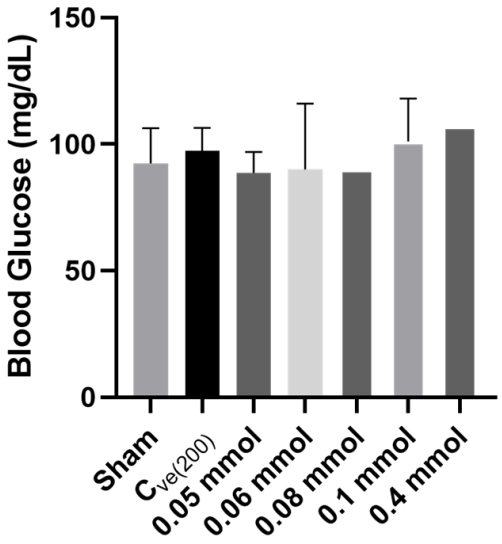


Figure 9: Blood glucose concentration of embryos after air sac injection of varying concentrations of D-Glucose at day 1. Results are presented as mean ± SD. n≥3

For more details, please refer to Annex 1 – table 19

In this sense, we searched the literature to try to find other approaches, and we came across two reports that used NaCl 0.72% as the vehicle solution, instead of CRS, and performed the injections on day 1 (Zhang et al., 2016) and (Tan et al., 2017).

Nonetheless, the data we obtained in the previous assays allowed us to select with some confidence the air sac injection maintaining the eggshell membrane (that displayed less mortality rate). Moreover, we opted to assess only two glucose conditions 0.2 and 0.4 mmol.

Viability assessment showed that in both controls, the embryos were mostly alive (Cve = 80% and Sham =71%). In the case of the two doses of glucose administered, the mortality rate increased significantly. In the case of D-Glucose 0.2 mmol embryos, 20% were registered as malformed and 64% of the embryos were dead. And, as expected, when the dose of glucose was doubled to 0.4 mmol, the death rate increased to 83% (Table 12).

(Zhang et al., 2016) showed that Sham group presented a 6.70% of mortality rate and 0% of gross abnormalities. For the vehicle group, the mortality increased to 10% and abnormalities were seen in 7.40% of the embryos. These results, although with values lower than ours, allow us to conclude that the procedure was successfuly performed.

For the glucose treated groups, (Zhang et al., 2016) reported 36.70% of dead embryos for the 0.2 mmol of glucose. (Tan et al., 2017) showed that in the 0.2 mmol group mortality rate was almost 40%

and in the 0.4 mmol group was 58.3 %. However, they described high percentages of malformations, around 50% for the 0.2 mmol group and 80% for the 0.4 mmol group. These results are not a perfect match to our data, but still, we decided to perform the glucose assessment.

Table 12: Effects of glucose air sac injection and NaCl 0.72% on day 1 on the percentage of embryo death and gross abnormality.

Experimental Condition	n	%Alive	%Malformed	%Dead
Sham	113	71	12	18
$Cve_{NaCl(200)}$	84	80	10	11
D-Glucose 0.2 mmol	133	16	20	64
D-Glucose 0.4 mmol	151	10	7	83

CveNaCl(200) where 200 is the volume injected in µL

Like in the previous tests, environment glucose was also assessed. It was possible to see a significant increase in glucose-treated eggs. The values of glucose increased from 136.70 mg/dL in the control group to 255.50 mg/dL in the first dose of glucose, 0.2 mmol, and 330.90 mg/dL in the dose of 0.4 mmol (Figure 10). These results allowed us to conclude that a hyperglycemic environment was being created however it was still necessary to confirm an increase in the blood glucose levels.

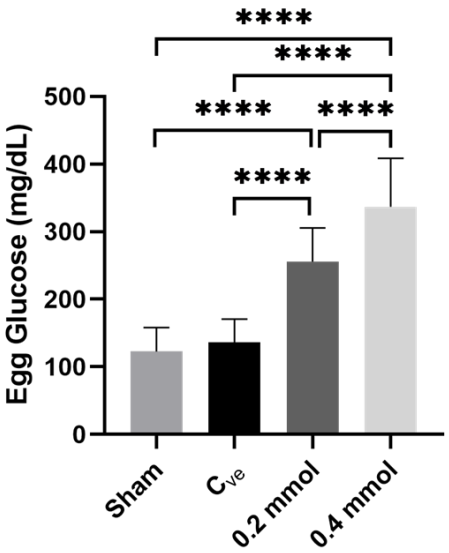


Figure 10: Egg environment glucose concentration of embryos after air sac injection of varying concentrations of D-Glucose at day 1. Results are presented as mean ± SD. ****$p <$ 0.0001. n≥29

For more details, please refer to Annex 1 – table 20

Blood glucose measurement revealed a significant difference between the control group and the doses administered (Figure 11). Blood glucose values rise from 85.80 mg/dL in the control to 94.06 mg/dL in 0.2 mmol dose and 95.40 mg/dL in 0.4 mmol dose.

Both, (Tan et al., 2017) and (Zhang et al., 2016), reported that the values of blood glucose rose from 80-90 mg/dL in control to approximately 120 mg/dL in the 0.2 mmol group and 140 mg/dL in the 0.4 mmol group. Despite this difference in the values, we obtained, for the first time, a significant increase in treated groups, meaning that we were able to induce hyperglycemia in the chicken embryo. The subsequent analyses were based on this approach.

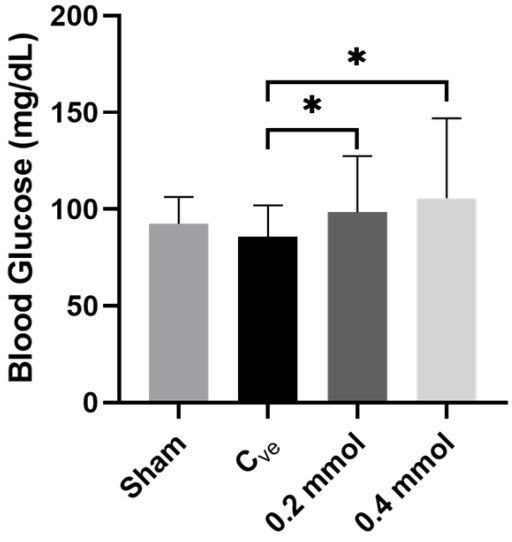


Figure 11: Blood glucose concentration of embryos after air sac injection of varying concentrations of D-Glucose at day 1. Results are presented as mean ± SD. *$p <$ 0.05. n≥29

For more details, please refer to Annex 1 – table 21

4.1.2. The effect of exogenous glucose injection procedure on embryo malformations

Exposure of the developing embryo to hyperglycemia can lead to devastating consequences, including spontaneous abortions, stillbirths, neonatal death, and congenital malformations. Malformations occur within the first 10 weeks of pregnancy during early organogenesis and almost any organ system can be affected (Loeken, 2020).

To determine if the exposure of the chicken embryo to exogenous and elevated glucose levels led to the development of severe malformations, a macroscopic analysis was performed. The administration of glucose caused severe malformations in the developing embryo, in a dose-dependent manner (Figure 12). C_{ve} and Sham embryos developed normally. On the contrary, embryos exposed to hyperglycemia presented defects in the optic organ, in the development of the brain (encephalocele, microcephaly, anencephalous), in the development of the neural tube (unclosed neural folds), and the heart (*Ectopia Cordis*, Cardiac Edema). It was also possible to observe an abnormal turning of the embryo in some embryos.

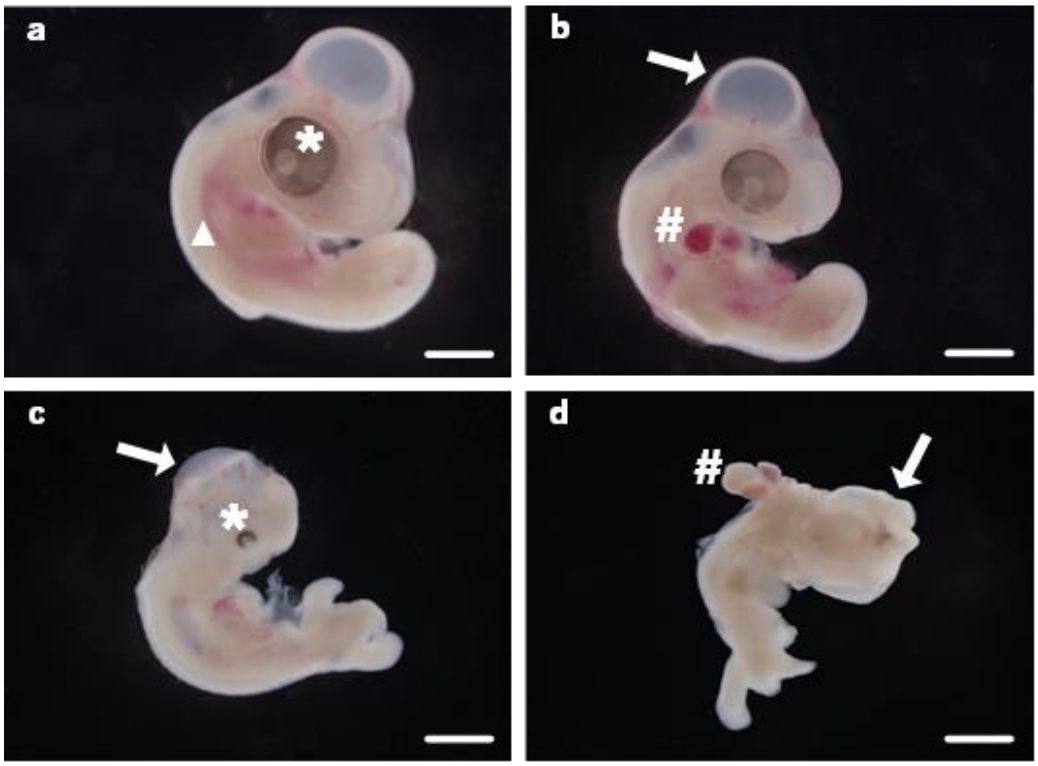


Figure 12: Developmental outcome of chicken embryos after D-Glucose exposure. a-Cve; b-Sham; c-0.2 mmol; d-0.4 mmol. Arrow: brain; Asterisk: eye; Arrowhead: aorta; Cardinal: heart; scale bar: 2000 μm.

To analyze these results more systematically, we developed a specific score based on the severity of the malformations. 53.8% of 0.2 mmol glucose embryo malformations and 66.7% of 0.4 mmol embryo malformations were scored as level 4, the highest level of this score. Additionally, 23.1% and 33.3% of 0.2 mmol and 0.4 mmol malformations, respectively, were classified as level 3. Finally, for the 0.2 mmol of glucose, 19.2% of the malformed embryos were categorized as a score level of 2 and 3.8% as a score level of 1. In the case of 0.4 mmol of glucose, there were no malformed embryos classified as level 1 or 2 (Figure 13).

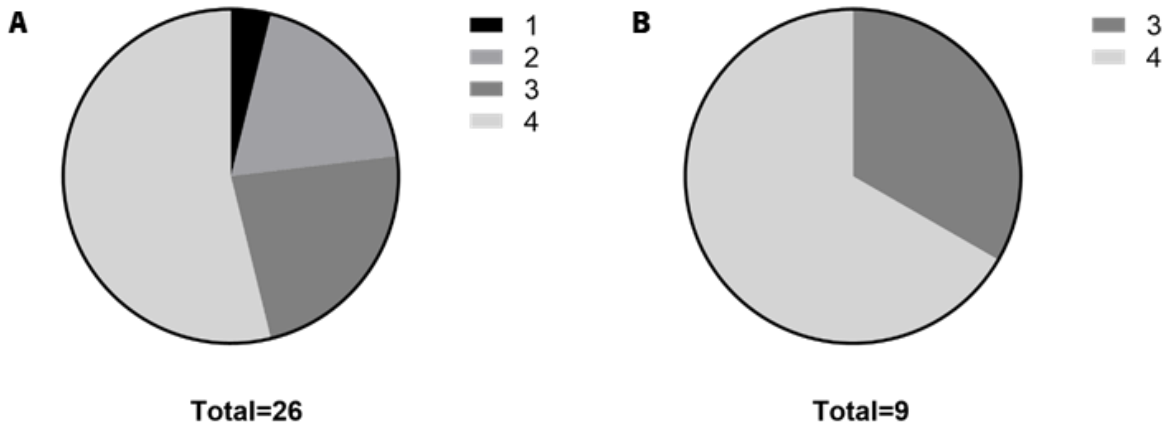


Figure 13: Statistical analysis of the levels of malformation. A- Score of malformations of 0.2 mmol embryos; B- Score of malformations of 0.4 mmol embryos.

A few studies in the literature also reported malformations in the developing embryo when exposed to high doses of glucose (Lawson et al., 2018; Tan et al., 2017; Zhang et al., 2016).

Lawson and coworkers demonstrated that hyperglycemia disturbed the normal development of the embryonic chicken heart, causing structural malformations in the endocardial cushions, two thicker areas that develop into the septum and in the outflow tract, a structure that connects the embryonic ventricles to the arterial system in the aortic sac. These malformations affect blood flow patterns, which may further enhance the teratogenic effects of hyperglycemia and contribute to secondary malformations in the vasculature (Lawson et al., 2018).

Additionally, it has been reported that a hyperglycemic state during chicken embryo development, leads to a high risk of stillbirth, growth retardation and development of congenital central nervous system (CNS) abnormalities similar to clinical manifestations, such as encephalocele, anencephaly, and exencephaly. The authors also showed that the incidence and severity of malformations increased in a dose-dependent manner (Tan et al., 2017).

Lastly, it has been shown that hyperglycemic conditions can induce osmotic stress, causing retina and lens cell lesions, in the chicken embryo. This study demonstrate that high glucose levels led to excess production of ROS, which, in turn, suppressed *pax6* expression, an important gene that regulates the formation of the optic vesicle, optic cup, lens placode and retina (Zhang et al., 2016).

Our results are in agreement with these studies and indicate that the hyperglycemic conditions induced in our model likely altered both the cardiac and nervous system development, causing the observed

malformations. Subsequently, in the second part of this work, we wanted to characterize the impact of hyperglycemia at the molecular level, since few studies have been performed in this area.

4.2. Molecular characterization of the impact of uncontrolled hyperglycemia on the expression levels of key glucose transporters and hormones in the liver

Glucose homeostasis must be tightly regulated, and the liver has a major role in controlling various pathways of glucose metabolism, including glycolysis. Glucose entrance into the hepatic cells occurs through transporters like GLUT1. GLUT2 acts as the primary glucose transporter and sensor in rodent pancreatic islets and is widely assumed to play a similar role in humans however recent findings showed that GLUT1 is more expressed in human pancreatic islets and beta-cells (McCulloch et al., 2011).

The insulin-like growth factor (IGF) system is an important regulator of growth and development in vertebrates. Throughout embryonic and postnatal development, and in adult life, the IGFs and their binding proteins are expressed in a wide variety of tissues. According to previous studies, IGF1 expression in the liver is undetectable until E19 (Liu, Guo, et al., 2016). In fact, during our conventional PCR studies, *igf1* was absent from the liver at E5 (Annex 1- Figure 16). Nonetheless, it was detected in the heart of chicken embryo (Annex 1- Figure 17), which might indicate that IGF1 may have a significant role in muscle tissue growth during chick embryo development. IGF2 has been shown to act in the muscles, helping to decrease blood glucose levels by facilitating glucose uptake, and to act on the embryonic liver to reduce hepatic glucose output and increase glucose storage as glycogen (Holly et al., 2019). In fact, the expression of IGF2 was detected in the chicken embryo liver on E10 with a peak at E14 (Liu, Guo, et al., 2016). Considering the major role played by GLUT1 and IGF2 in liver/glucose homeostasis, we asked whether they were affected by the induction of hyperglycemia. In this sense, mRNA expression levels of *igf2* and *glut1* in the liver from live embryos were quantified by qPCR. Results showed a clear increasing tendency in a dose-dependent manner in *igf2* expression levels; however, only the 0.4 mmol dose displayed a statistically significant difference with the Sham group. On the other hand, *glut1* expression levels decreased in a dose-dependent manner, presenting a statistically significant difference with the control group (Figure 14).

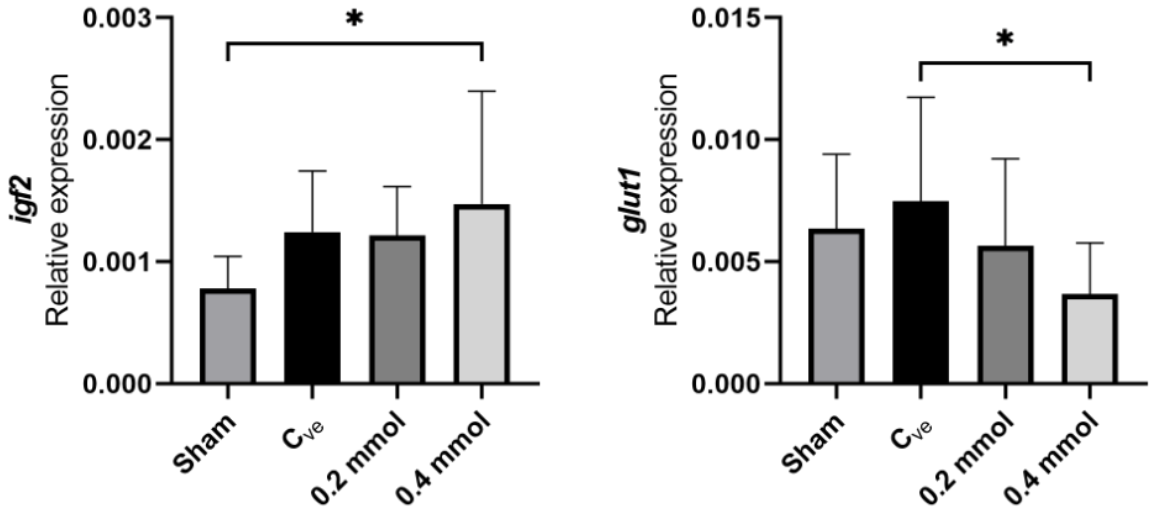


Figure 14: The effect of exogenous glucose on hepatic *igf2* and *glut1* expression. Results are presented as mean ± SD, normalized for β-actin. $*p < 0.05$. n≥6

Although IGF2 plays a fundamental role in embryonic development in mammals, understanding its physiological and pathological role is limited compared with IGF1 (Dupont & Holzenberger, 2003). Furthermore, a few studies have been conducted in avian species to study the expression of insulin-like growth factor system genes; moreover, the early embryonic period (E0 to E5) has not been extensively studied until today (Liu, Guo, et al., 2016; Liu, Zhi, et al., 2016; Lu et al., 2007; McMurtry et al., 1998; Mohammed et al., 2017; Richards et al., 2005). Some studies, predominantly in mammals, have evaluated IGF2 during a diabetic state (Imai et al., 2010; Sireesha et al., 2009; Xuan et al., 2019). It has been shown that IGF2 levels increase in the liver of diabetic mice (Ge et al., 2018). Indeed, we observed an increase in *igf2* expression levels in hyperglycemic livers from chicken embryos. Since there is more glucose in the system, it needs to be properly metabolized by the liver by boosting the storage of glycogen. It seems an expected response considering the over-availability of glucose.

On the other hand, GLUT1 has been widely studied in healthy livers and in metabolic disorders (Chadt & Al-Hasani, 2020; Meireles et al., 2017). In 1995, the presence of GLUT1 was characterized for the first time in chicken embryo fibroblasts (Wagstaff et al., 1995). Since then, some studies have described the alterations in GLUT1 in a hyperglycemic state. It has been reported that after D-Glucose administration, *glut1* expression decreased in the whole chicken embryo (Tan et al., 2017) and the chicken embryonic eye (Zhang et al., 2016). We detected a significant decrease in *glut1* expression in chicken embryo hyperglycemic livers. This downregulation is probably a result of cellular protective mechanisms under the unfavorable high-glucose environment.

Additionally, a study in a GLUT1 knockout mice revealed that the suppression of GLUT1 lead to the development of embryonic malformations like caudal regression and anencephaly with absence of the head. These malformations are similar to those observed in embryos exposed to the hyperglycemia of maternal diabetes where embryonic GLUT1 is known to be reduced (Heilig et al., 2003).

In conclusion, even though only the highest dose of glucose administrated revealed significant alterations in expression of these genes, our results are in accordance with the literature and further validate our approach as a successful method to induce a hyperglycemic state during early embryo development.

4.3. Impact of hyperglycemia in the modulation of oxidative stress

Hyperglycemia activates a particular metabolic route that involves diacylglycerol (DAG)—protein kinase C (PKC)—and NADPH-oxidase, culminating in the production of Reactive Oxygen Species (ROS). This pathway has been considered a "dangerous metabolic route in diabetes" because, an increase in oxidizing species in the absence of an antioxidant response, a consequence of hyperglycemia, leads to a state of oxidative stress. This condition can disrupt various signaling pathways that may result in the onset and progression of complications, such as vascular dysfunction and pathologies (Nogueira-Machado & Chaves, 2008). Conversely, superoxide dismutases (SODs) are a group of metalloenzymes that constitute a very important antioxidant defense against oxidative stress in the body (Landis & Tower, 2005). These enzymes catalyze the conversion of superoxide(O_2^-) into oxygen and hydrogen peroxide(H_2O_2) (Younus, 2018), therefore controlling the levels of ROS and limiting the potential toxicity of these molecules. For this reason, we decided to measure SOD activity in the whole embryo. Specifically, we used normal control versus malformed treated embryos. Results showed that, in a hyperglycemic state, the levels of SOD significantly decrease in glucose-treated embryos when compared to both control groups (Figure 15).

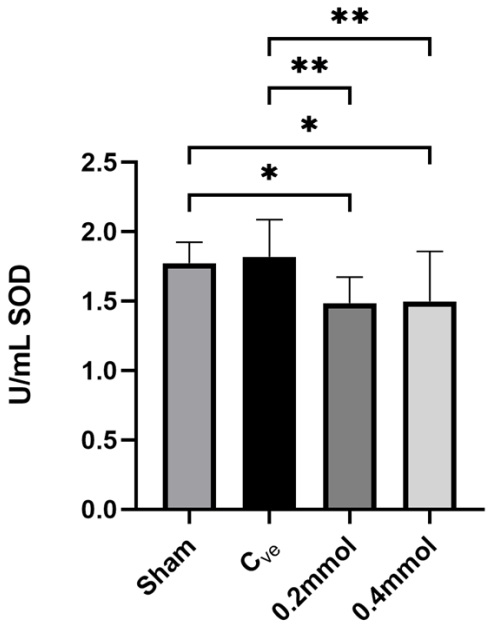


Figure 15: SOD activity quantification in embryos. Results are presented as mean ± SD. *$p < 0.05$; **$p < 0.01$. n≥6

During early pregnancy, the embryo uses high levels of oxygen, and because its antioxidant defenses are not well developed, the embryo becomes vulnerable to oxidative damage (Jin et al., 2013). The elevation of ROS during oxidative stress has long been linked to diabetes or diabetic pregnancies (Eriksson & Borg, 1993; Wender-Ozegowska et al., 2004).

Alternatively, elevated levels of SOD are shown to lower oxidative stress; however, in diabetic tissues, several studies reported a decline in these levels (Fujita et al., 2009; He et al., 2011; Skrha et al., 1996). Zhang and co-workers showed that embryos treated with D-Glucose presented a higher production of ROS in the eye tissue and decreased in SOD activity levels (Zhang et al., 2016). In our case, we did not analyze a specific tissue; instead, we evaluate whole embryos from severely malformed chicken embryos. Despite that, our results also showed a decrease in the levels of SOD activity, which means that the developing embryos are exposed to a high level of oxidative stress, thus contributing to unfavorable outcomes for the embryo.

Chapter 5. Conclusions and Future Perspectives

It is well established that the first weeks of pregnancy are crucial for the proper development of the internal organs of the embryo. Insults during this period, like hyperglycemia, may lead to severe developmental abnormalities or congenital malformations.

In this work, we retrieved the chicken embryo model as a model to characterize the direct impact of hyperglycemia on organogenesis. Although this model had been employed in the past by some authors, it is not widely used. After several optimization procedures, and based on the available literature, we were able to establish a reproducible method to induce hyperglycemia during early embryonic development. Our results showed that a hyperglycemic state resulted in severe malformation of the embryos and a high mortality rate. Malformations were observed in the eye, brain, heart, and neural tube. Furthermore, the increased expression of *igf2* and decrease of *glut1* in the liver, proved that despite the lack of insulin to regulate glucose metabolism, the embryo successfully initiates a defense mechanism against the high glucose levels in circulation. Finally, the decrease in SOD values may be contributing to the teratogenic effect of glucose.

Further studies are necessary to fully characterize this model for the study of diabetes in pregnancy, for example, perform an ELISA to corroborate the insulinemic and IGF2 profile of the embryo.

In the future, it would be interesting to evaluate the impact of hyperglycemia in a major organ that is yet to be studied during the embryonic phase, the lung. Namely, to perform a morphometric analysis to detect if hyperglycemia impairs lung growth and branching. Also, to determine whether the expression of molecular players involved in patterning and branching would be impaired in lungs exposed to uncontrolled hyperglycemia. Furthermore, it would be interesting to evaluate glucose catabolism genes to uncover the impact of hyperglycemia in lung metabolism and insulin/IGF receptors. Finally, it would be interesting to uncover the impact on oxidative stress by analyzing members of the ROS pathway.

Chapter 6. References

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Annex 1

Table 13: Values of blood glucose concentration of embryos after yolk injection of varying concentrations of D-Glucose at day 0

Experimental Condition	n	Mean ± S.D.	Minimum	Maximum
Sham	4	87.00 ± 20.18	58	103
$Cve_{CRS\ (300)}$	11	96.64 ± 10.66	78	118
$Cve_{CRS\ (450)}$	9	88.44 ± 3.97	82	94
$Cve_{CRS\ (600)}$	3	83.00 ± 6.93	79	91
D-Glucose 0.03 mmol	5	84.40 ± 4.28	78	90
D-Glucose 0.3 mmol	9	94.25 ± 11.89	76	111
D-Glucose 0.45 mmol	10	103.70 ± 4.66	98	112
D-Glucose 0.6 mmol	5	87.75 ± 3.86	82	90
D-Glucose 1.2 mmol	5	101.00 ± 6.56	92	108

CveCRS(xxx) where xxx is the volume injected in µL

Table 14: Values of blood glucose concentration of embryos after air sac injection, without membrane, of varying concentrations of D-Glucose at day 0.

Experimental Condition	n	Mean ± S.D.	Minimum	Maximum
Sham	3	96.33 ± 9.29	86	104
$Cve_{CRS\ (250)}$	2	109.50 ± 2.12	108	111
D-Glucose 0.375 mmol	1	154.00 ± 0.00	154	154
D-Glucose 0.5 mmol	1	105.00 ± 0.00	105	105

CveCRS(250) where 250 is the volume injected in µL

Table 15: Values of blood glucose concentration of embryos after air sac injection, without membrane, of varying concentrations of D-Glucose at day 1.

Experimental Condition	n	Mean ± S.D.	Minimum	Maximum
Sham	1	118.00 ± 0.00	118	118
$Cve_{CRS\ (200)}$	2	100.50 ± 12.02	92	109
D-Glucose 0.4 mmol	1	106.00 ± 0.00	106	106

CveCRS(200) where 200 is the volume injected in µL

Table 16: Values of egg glucose concentration of embryos after air sac injection, with membrane, of varying concentrations of D-Glucose at day 0.

Experimental Condition	n	Mean ± S.D.	Minimum	Maximum
Sham	39	129.50 ± 25.11	85	203
$Cve_{CRS\ (200)}$	37	132.70 ± 30.54	73	203
D-Glucose 0.1 mmol	32	156.00 ± 35.70	85	241
D-Glucose 0.15 mmol	35	160.70 ± 38.84	101	251
D-Glucose 0.2 mmol	32	204.90 ± 37.42	124	280
D-Glucose 0.3 mmol	33	241.00 ± 43.21	150	310
D-Glucose 0.4 mmol	29	274.70 ± 53.51	154	353

CveCRS(200) where 200 is the volume injected in µL

Table 17: Values of blood glucose concentration of embryos after air sac injection, with membrane, of varying concentrations of D-Glucose at day 0.

Experimental Condition	n	Mean ± S.D.	Minimum	Maximum
Sham	17	102.80 ±10.16	89	128
$Cve_{CRS\ (200)}$	21	94.71 ± 18.73	49	131
D-Glucose 0.1 mmol	15	98.93 ± 6.99	84	109
D-Glucose 0.15 mmol	17	100.10 ± 9.31	90	117
D-Glucose 0.2 mmol	5	91.00 ± 9.35	84	107
D-Glucose 0.3 mmol	5	112.30 ± 6.40	105	119
D-Glucose 0.4 mmol	3	97.33 ± 5.51	92	103

CveCRS(200) where 200 is the volume injected in µL

Table 18: Values of egg environment glucose concentration of embryos after air sac injection, with membrane, of varying concentrations of D-Glucose at day 1.

Experimental Condition	n	Mean ± S.D.	Minimum	Maximum
Sham	29	122.70 ± 35.21	35	188
$Cve_{CRS\ (200)}$	14	118.80 ± 31.39	68	173
D-Glucose 0.05 mmol	4	127.00 ± 24.99	90	145
D-Glucose 0.06 mmol	7	143.00 ± 39.60	86	209
D-Glucose 0.08 mmol	5	132.60 ± 25.23	107	167
D-Glucose 0.1 mmol	5	151.40 ± 30.74	111	184
D-Glucose 0.4 mmol	3	266.30 ± 20.43	243	281

CveCRS(200) where 200 is the volume injected in µL

Table 19: Values of blood glucose concentration of embryos after air sac injection, with membrane, of varying concentrations of D-Glucose at day 1.

Experimental Condition	n	Mean ± S.D.	Minimum	Maximum
Sham	43	92.40 ± 13.91	59	122
$Cve_{CRS\ (200)}$	24	97.38 ± 9.03	87	119
D-Glucose 0.05 mmol	3	88.67 ± 8.33	82	98
D-Glucose 0.06 mmol	5	90.00 ± 26.12	47	112
D-Glucose 0.08 mmol	1	90.00 ± 0.00	90	90
D-Glucose 0.1 mmol	3	101.00 ± 17.00	84	118
D-Glucose 0.4 mmol	1	107.00 ± 0.00	107	107

CveCRS(200) where 200 is the volume injected in µL

Table 20: Values of egg environment glucose concentration of embryos after air sac injection, with membrane, of varying concentrations of D-Glucose at day 1.

Experimental Condition	n	Mean ± S.D.	Minimum	Maximum
Sham	29	122.70 ± 35.21	35	188
$Cve_{NaCl(200)}$	33	136.70 ± 33.80	58	227
D-Glucose 0.2 mmoL	36	255.50 ± 50.00	150	378
D-Glucose 0.4 mmol	35	330.90 ± 62.04	183	456

CveNaCl(200) where 200 is the volume injected in µL

Table 21: Values of blood glucose concentration of embryos after air sac injection, with membrane, of varying concentrations of D-Glucose at day 1.

Experimental Condition	n	Mean ± S.D.	Minimum	Maximum
Sham	43	92.40 ± 13.91	59	122
$Cve_{NaCl(200)}$	35	85.80 ± 16.16	47	115
D-Glucose 0.2 mmoL	32	94.06 ± 13.93	59	121
D-Glucose 0.4 mmol	20	95.40 ± 26.19	52	156

CveNaCl(200) where 200 is the volume injected in µL

Annex 2

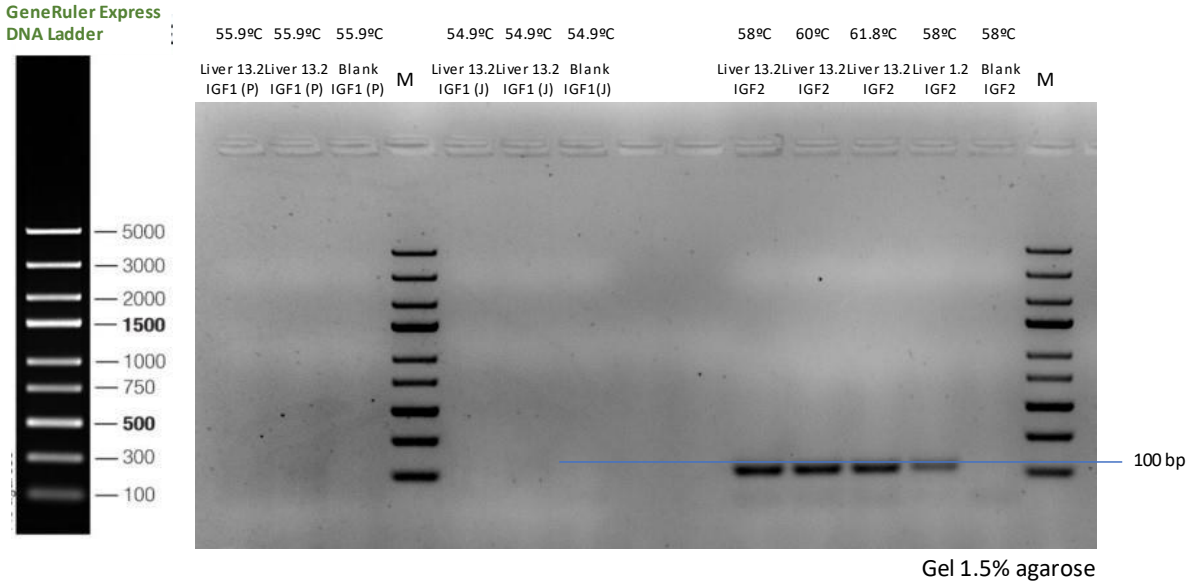

Figure 16: Conventional PCR, with different annealing temperatures, for *igf1* and *igf2* gene in the liver of chicken embryos. M- DNA molecular weight ladder (MassRuler DNA Ladder Mix,ThermoFisher).

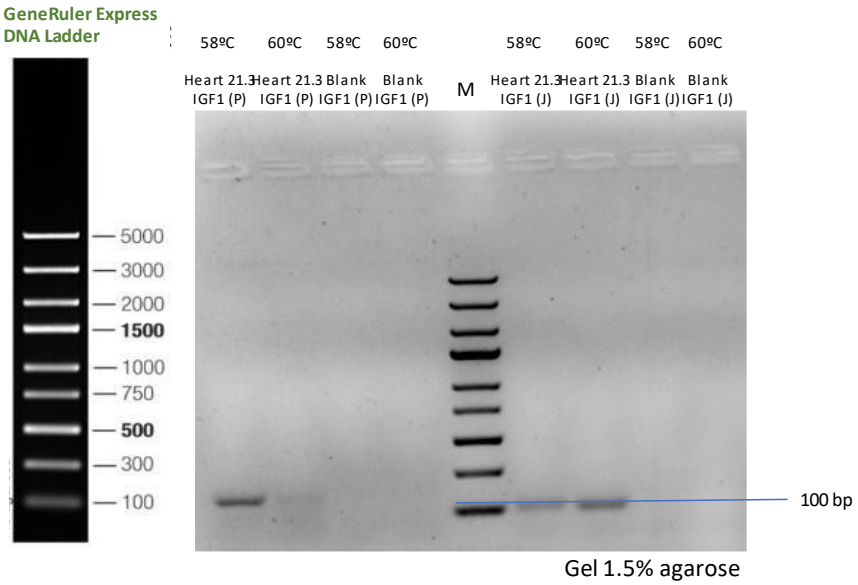

Figure 17: Conventional PCR, with different annealing temperatures, for *igf1* gene in the heart of chicken embryos. M- DNA molecular weight ladder (MassRuler DNA Ladder Mix,ThermoFisher).