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REVIEW

Modeling Chick to Assess Diabetes Pathogenesis and Treatment

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■ Abstract

Animal models have been used extensively in diabetes research. Studies on animal models have contributed to the discovery and purification of insulin, development of new therapeutic approaches, and progress in fundamental and clinical research. However, conventional rodent and large animal mammalian models face ethical, practical, or technical limitations. Therefore, it would be beneficial developing an alternative model for diabetes research which would overcome these limitations. Amongst other vertebrates, birds are phylogenically closer to mammals, and amongst birds, the chick has been used as one of the favored models in developmental biology, toxicology, cancer research, immunology, and drug testing. Chicken eggs are readily available, have a short incubation period and easily accessible embryos. Based on these inimitable advantages, the present review article aims to discuss the suitability of the chick as a model system to study specific aspects of diabetes. The review focuses on the application of *i*) chick pancreatic islets for screening of antidiabetic agents and for islet banking, (*ii*) shell-less chick embryo culture as a model to study hyperglycemia-induced malformations observed in mammalian embryos, and (*iii*) chick chorioallantoic membrane (CAM) to examine glucose-induced endothelial damage leading to inhibition of angiogenesis.

Keywords: diabetes · chick islets · chick embryo · chorioallantoic membrane · ethical consideration · endocrine pancreas · beta-cell · insulin secretion

Introduction

Diabetes is a multifactorial metabolic disorder which has reached epidemic proportion all over the world. The focus of diabetes research is the unraveling of diabetes pathogenesis and the discovery of novel drugs which are able to stimulate or enhance insulin secretion, control hyperglycemia, and reduce the occurrence of secondary complications of the disease. Many diabetic patients exhibit poor glycemic control, others fail to respond to the treatment, and some develop serious complications. Therefore, more effective treatment options for diabetes are needed [1].

The rising incidence of gestational diabetes across the industrialized world is largely paralleled by the increased prevalence of obesity. Simultaneously, there has been a sharp increase in the risk of pregnancy complications related to the birth of macrosomic babies. The associated long-term complications of gestational diabetes indicate a future rise in the incidence of type 2 diabetes and associated conditions in both the mother and her affected offspring [2].

A wide range of mammalian models has been inbred for many generations by selecting for hyperglycemia. Among them are the non-obese diabetic (NOD) mouse and the BioBreeding (BB) rat, which spontaneously develop the disease with similarities to human type 1 diabetes. These models are routinely used as mammalian models for studying human type 1 diabetes.

The Goto Kakizaki (GK) rat is developed by the selective breeding of Wistar rats with the highest blood glucose over many generations. These rats develop relatively stable hyperglycemia in adult life. Fasting blood glucose is only mildly elevated, but rises on challenge with glucose. Both insulin resistance and impaired insulin secretion are present. This model is used for the study of human type 2 diabetes. Virus or chemically induced diabetic animals have also been developed to study different aspects of diabetes. Pregnant mammals and isolated islets from various species of mammals have formed effective *in vivo* and *in vitro* models for understanding the pathophysiology of gestational diabetes and for screening hypoglycemia, respectively [3, 4].

Today, animal experimentation is often contentious and subject to legal and ethical restrictions that vary throughout the world. In most countries, experiments with animals, especially with mammals, need ethics committee approval. Alternatives to mammalian models could facilitate experiments.

Beside mammals, poultry is used as a model for studying atherosclerosis, hypertension, and cholesterol metabolism. Poultry is also used in bone development, pathology, and surgical studies. These models have made a tremendous progress in biomedical research. In particular the chick can directly reflect the human condition, as shown in spinal cord repair or in chorioallantoic membrane wound healing [5]. Therefore, it is able to act as an *in vivo* model for studying repair process. It is also an established model for tissue/cell transplantation. Because of its lack of immune system in early developmental stages, it is increasingly recognized as a potential model for mammalian biology with new applications for stem cell and cancer research [6].

Based on these findings, the chick can be considered as a model for regenerative therapy [7]. Organ culture studies on glucose, glucagons, and tolbutamide stimulation of chick (grainivorous bird) endocrine pancreas have suggested similarities in avian and mammalian beta-cell insulin secretory mechanisms [8-10]. Reversal of experimental diabetes in mice by embryonic chick pancreatic transplants has also been reported [11]. However, its potential as a model for diabetes research has often been overlooked. Work from our lab has shown that isolated chick islets are suitable for screening of hypoglycemic agents and for islet banking (Figure 1) [12, 13]. Our work has also revealed that the shell-less chick embryo culture system can be used for studying glucose-induced malformations similar to those observed in mammalian embryos [14].

The aim of the present review is to discuss the potential of the chick as a model to assess specific aspects of diabetes research. It is also intended to show how the chick model can be utilized to address hyperglycemia-related impairments affecting islets, embryo development, and angiogenesis. Therefore, this review can be regarded as a starting point for specific aspects of animal-based research into diabetes. Our review does not argue that chick models are better than mammalian models, but emphasizes the advantages of the chick as a suitable substitute in selected experimental investigations.

Abbreviations:
AGE - advanced glycation end product
BB - BioBreeding
CAM - chorioallantoic membrane
DMEM - Dulbecco's modified Eagle's medium
FCS - fetal calf serum
FPG - fasting plasma glucose
GK - Goto Kakizaki
HH6 - head-fold stage
NOD - non-obese diabetic
PG - plasma glucose
ROS - reactive oxygen species
STZ - streptozotocin

Diabetes in mammals and birds

In mammals, diabetes is a metabolic syndrome characterized by hyperglycemia, with plasma glucose (PG) of ≥ 200 mg/dl, while normal fasting plasma glucose (FPG) is below 110 mg/dl, and 2-hour PG is below 140 mg/dl. Sedentary habits and obesity result in diminished sensitivity to glucose and insulin, and finally in insulin resistance. Hepatic glucose production may continue even if plasma glucose and insulin are both elevated. Insulin production by beta-cells fails to compensate for decreased insulin sensitivity, which results in hyperglycemia [15, 16]. Secondary complications of diabetes include diabetic retinopathy, nephropathy, and neuropathy. Arteriosclerosis is accelerated by diabetes. Microangiopathy, macroangiopathy, diabetic angiopathy, diabetic foot disease, diabetic ketoacidosis, and dyslipidemia are further abnormalities occurring with progression of diabetes in mammals [17].

In contrast to mammals, diabetes is rarely diagnosed in birds, mainly because of the highly variable (210-550 mg/dl) normal blood glucose values observed in avian species [18]. The combination of high levels of glucose and free fatty acids,

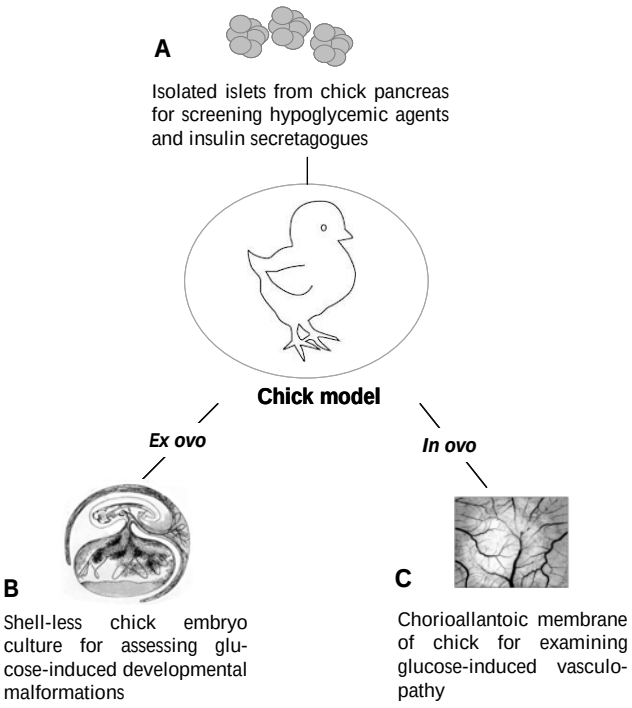

Figure 1. The chick model for different aspects in diabetes research. A: Dithiazone (DTZ)-stained functional islets isolated from the dorsal lobe of the pancreas of a 4-5 days old chicken are suitable for screening for antidiabetic agents. **B**: Chick embryo shell-less culture model for studying glucose-induced malformations in mammalian embryos. **C**: Chick chorioallantoic membrane (CAM) as a model to study glucose-induced inhibition of angiogenesis.

normal to high levels of insulin, high glucagon levels, and insulin resistance in birds has indicated similarities between their normal condition and maturity onset diabetes in man [19]. The majority of reported cases of avian diabetes have occurred in small birds such as the parakeet and canary [18]. The clinical signs of diabetes in birds are similar to those in mammals and include polyuria, polydipsia, polyphagia, and weight loss [20]. Clinical pathology findings are also similar and include persistent hyperglycemia, which is in the range of 500-1800 mg/dl and glucosouria. Reported etiologies include pancreatic neoplasia, pancreatitis, pancreatic atrophy, and idiopathy [21, 22].

In avian species, it is glucagon overproduction that causes the blood-sugar level to rise, whereas insulin deficiency or insulin resistance is the cause of mammalian diabetes [23]. However, the relative importance of insulin and glucagon in glucose metabolism and pathogenesis of diabetes in birds are incompletely understood [20]. Hummingbirds and many other migratory birds maintain very high glucose and elevated body fat as an adaptive trait without developing diabetes [24-26]. These birds consume high sugar food (nectar) and accumulate over 40% body fat shortly before migration, but they do not develop diabetes, polyurea, polyphagia, or polydipsia [27-29]. They have a very high metabolic rate and lose most of the stored fat in 20 hours by flying up to 600 miles across the Gulf of Mexico. Hummingbirds can quickly enter into torpor, and reduce resting metabolic rates 10 fold. They combat kinetic disorders by maintaining close feedback between energy intake and energy expenditure. Thus, this bird species may offer lessons for the prevention of diabetes and obesity [24].

In spite of these lifestyle and metabolic differences, similarities have been reported between avian and mammalian insulin secretory mechanisms [8-10], and the structure of insulin [8-10, 30]. Unlike rodents which have two pre-proinsulin genes [30], humans and chickens have a single pre-proinsulin gene, indicating similarities in prepancreatic expression characteristics [31]. It has been also reported that the organization of the human genome is closer to that of the chicken than that of the mouse [32]. This review highlights the similarities and differences between chick and human/mammal, and introduces the chick as *in vitro* and *in ovo* model for investigating diabetic pathogenesis, embryo development, and islet function.

Usability of chick pancreatic islets to screen for insulin secretagogues

Diabetes is the manifestation of complex pathophysiological interactions between hyperglycemia, beta-cell dysfunction, insulin resistance, dyslipidemia, and endothelial cell dysfunction. Experimental diabetic mammals and mammalian pancreatic islets have been routinely used for *in vivo* and *in vitro* studies with respect to different aspects of diabetes [33-37]. There are no reports on the use of isolated islets from other vertebrates for the same purpose. There are few reports on islet isolation from the chicken pancreas [38, 39].

The chicken pancreas is an elongated, lobed gland, located in the duodenal loop, and characterized by three types of islets, namely A, B, and mixed islets. The light or B islets (beta islets) consist of beta-cells and alpha1-cells. The dark or A islets (alpha islets) consist predominantly of alpha2-cells and alpha1-cells, while mixed or

'mammalian type' islets are composed of numerous beta-cells and only a few alpha- and delta-cells. The A and mammalian type islets are almost exclusively confined to the splenic and third lobes, whereas B islets are distributed throughout all the lobes of the pancreas [40, 41].

High levels of normal blood glucose in birds and insufficient insulin response to glucose by islets from 4- to 6-week-old chicks have been reported [19]. However, our blood glucose measurements in embryonic chicks up to 15 days after hatching [12], and previous studies on metabolic hormone profile in embryonic and posthatch chicks [42], have shown a gradual increase in blood glucose levels [9, 43, 44]. Therefore, we examined glucose response and function of B islets isolated from 5-6 day old chick pancreas [45]. In contrast to the previous reports, we found that these islets are responsive to insulin secretagogues, such as glucose, arginine, toulbutamide and others. This finding suggests that these islets could be used for screening of antidiabetic agents, as an alternative to mammalian islets [12].

Chick and human pancreatic islets are insensitive to the known diabetogen streptozotocin (STZ) [46-49]. Therefore, experimental diabetes in chicks must be provoked other than in mammals. We found that the stronger antioxidant defense mechanism and higher uric acid contents of chick islets counteract STZ-induced ROS generation and render chick islets insensitive to STZ [50]. Further studies are required to understand the insensitivity of human islets to STZ. Our comparative studies on STZ treatment in chick and mouse islets suggest that chick islets can be used as a model for diabetes research to confirm the role of antioxidant defense mechanisms in counteracting oxidative stress [50].

There are many studies on isolation and cryopreservation of mammalian islets [51-56], but reports on cryopreservation of chick islets are lacking. We attempted to cryopreserve isolated chick islets following an established protocol used for cryopreservation of mouse islets [57]. The islets were cryopreserved in a medium consisting of DMEM:Ham's F.12 (1:1) (Gibco, Brazil), supplemented with 10% FCS, riboflavin, and nicotinamide as cryoprotectants [58-64]. We found that it is possible to cryopreserve chick islets without losing viability and insulin secretory activity [13]. This finding confirms the usability of chick pancreatic B islets as an *in vitro* model for physiological and pharmacological studies in mammalian and poultry diabetes.

Studies have also reported the effectiveness of xenogeneic embryonic tissue in the treatment of experimental diabetes in rats [11]. Approximately eighty splenic lobes of pancreas obtained from 15 to 18 days old chick embryos, composed almost exclusively of endocrine tissue, were implanted directly into the hepatic parenchyma of the rat recipient. The biochemical and metabolic changes in the recipients suggested that embryonic transplants of 15 days old chick pancreases were able to significantly improve the hyperglycemic state of rats, for a prolonged period of time (18 months) and without any immunosuppression. Based on these findings, it is worth attempting transplantation of isolated islets from the chick pancreas 4-5 days posthatch in experimental diabetic animals to examine whether it leads to diabetes reversal without immunosupression. Since the ratio of insulin to glucagon cells is decreased in the avian pancreas, the condition is similar to that in type 2 diabetes [65] Further experimentation with the chick pancreas is necessary to investigate the scenario existing in type 2 diabetes.

Chick embryo as a model for depicting glucose-induced malformations

Chick embryo is easily accessible, has a well-established development pattern, and is isolated from maternal influences [66]. It is one of the most versatile *in vivo* models used for various experimental settings. The easy availability of eggs and the rapid development of chick embryos has led to the development of various methods of chick embryo culture [67, 68]. These include window technique [69], *in vitro* culturing of embryos on a glass ring in albumen [70], and *ex ovo* culture systems [71-75]. The Hamburger-Hamilton staging system for chicks [76] and the Carnegie stages of development, which reveal the correlation of chick development with almost every other placental mammal [77], provide a useful database for employing chick embryo as a model system. Since developing chick embryos are not considered to be animals for up to ten days of embryonic life [78], its use in experiments does not evoke serious ethical issues. Therefore, the chick embryo model, produced by various culture methods, has been routinely used as a model for developmental biology, toxicology, cancer research, and immunology.

Teratogenic effects of high glucose and very high insulin on chick embryo have been documented. It has been also shown that the severity of malformations depends on the stage of devel-

opment [79]. Given the inaccessibility of embryos in mammalian models, which limits *in vivo* experiments [80], studies have used neurulating chick embryos to document similarities between chick and mammals in the role of insulin in growth acceleration and differentiation during embryonic development [81, 82]. These studies have shown that the neurulating chick embryo is an excellent alternative model to study endogenous insulin signaling and its contribution to early embryonic cell survival [81, 82]. However, experimental failures and limited embryo growth have hampered the wide use of this chick embryo model. Therefore, chick embryo was not recommended as a model for diabetes research.

Gestational diabetes is known to increase the risk of congenital malformation and birth defects in offspring compared to the children of non-diabetic women. Malformations associated with diabetic pregnancy affect many major organ systems, such as the central nervous, cardiovascular, gastrointestinal, urogenital, and musculoskeletal systems [83]. The infant of the diabetic mother has an increased risk for several neonatal complications, such as macrosomia, hypoglycemia, hypocalcemia, polycythemia, and hyperbilirubinemia. Up to 25% of such children have been reported to be afflicted with these complications [84]. Studies in humans, exploring the mechanisms of these alterations, are limited by ethical reasons and by the multiplicity of uncontrolled variables which may modify the intrauterine environment and cause potential effects on congenital malformations [85]. Therefore, there is a need for appropriate animal models. As chick is phylogenically closer to mammals, and offers the unique advantages discussed above, it can act as an alternative model for gestational diabetes.

Chick embryo culture systems, as reported earlier, either support the development of chick embryo from the early stages (HH6) to a few hours (48 hr) or from 48 hr to a few days. We have developed a simple shell-less chick embryo culture system [14], which supports the culturing of chick embryos from the very early stages of morphogenetic movements through the stages of active organogenesis to the seventh day of incubation, outside the eggshell in a glass bowl. This culture system is economical and highly reproducible. The effects of hyperglycemic glucose concentrations on various stages of active organogenesis in chick embryo were studied using this culture system. It was observed that the effects depended on concentration and developmental stage, and that they were comparable to those reported for mouse, rat, and human embryos. Different diabetic malformations of mammalian gestational diabetes, such as embryonic dismorphogenesis, rotational defects, cardiovascular abnormalities, macrosomia, macrocephaly, and retarded growth could be observed using this system. This study indicated that the developing chick embryo provides an excellent alternative model system to mammalian embryo models for the study of glucose-induced malformations [14]. The shell-less chick embryo culture system can also be employed in biomedical research for exploring the effects of angiogenic agents, vitamins, antibiotics, viruses, and carcinogenic agents.

Chorioallantoic membrane (CAM) of chicks for assessing the effect of high glucose on angiogenesis

Angiogenesis refers to the formation of new blood capillaries that develop from existing ones [86, 87]. It is essential in normal developmental processes and in numerous pathologies, including diabetic retinopathy, psoriasis, and tumor growth. A variety of *in vivo* and *in vitro* angiogenesis assay systems have been developed to test the efficacy of a wide range of both pro- and anti-angiogenic agents [88].

The chick chorioallantoic membrane (CAM) assay is probably the most widely used *in vivo* assay for studying angiogenesis [89, 90]. The test substance is placed in various ways onto the CAM, either through a window cut carefully in the eggshell or directly onto the CAM *in vitro*. The lack of a mature immune system in 7-8 days old chick embryos means that tumor-induced angiogenesis can be studied [91]. The angiogenic effects can be measured by counting the number of blood vessels in a given area using a stereomicroscope [92].

Diabetes is associated with abnormal angiogenesis. Increased angiogenesis contributes to severe forms of diabetic retinopathy, but angiogenesis is decreased in response to myocardial ischemia in diabetic patients. In most cases, angiogenesis is impaired in diabetic patients, a situation which contributes to impaired wound healing. In case of solid organ transplantation, outcomes can be worse than in non-diabetic patients [93].

The direct effect of hyperglycemia on angiogenesis can be evaluated in the chicken CAM assay, a model of active neoangiogenesis. Hyperglycemia induces defects in angiogenesis without alteration in the expression of major vascular growth factors in the chicken CAM model. A direct negative effect of hyperglycemia on angiogenesis

may be responsible for failures of "therapeutic angiogenesis" trials. Using this model, it has been shown that a modest degree of hyperglycemia decreases angiogenesis by increasing apoptosis and decreasing proliferation of vessel wall cells. The same CAM model has also been explored by investigating the capacity to build an angiogenic response to proangiogenic factors in hyperglycemic conditions [94, 95]. Various mechanisms have been postulated to explain the impaired angiogenic response in diabetes:

1. The presence of vascular dysfunction characterized by both endothelial and vascular smooth muscle impairments [96].
2. Exposure to chronic hyperglycemia which leads to nonenzymatic glycation of proteins and impaired formation of new blood vessels.
3. The presence of diabetes is associated with abnormalities in growth factor signaling [97] and/or expression [98], disturbing the local balance of vascular growth factors.

Another critical complication of diabetes is the formation of advanced glycation end products (AGEs) which have been associated with the development of diabetic microangiopathy. To date however, the effects of AGEs on angiogenesis have generally been examined either *in vitro* or by using cultured cells. Using the CAM assay, it has been shown that AGEs induce angiogenesis *in vivo*. Immunohistochemical analysis demonstrated that AGE-induced vascular lumens were devoid of pericytes. This study further confirmed that AGEs themselves were angiogenic in nature, and that AGE induced abnormal vessels in CAMs. These findings confirm that CAM is a useful model for investigating diabetic retinal angiogenesis, and that it can be used to provide a therapeutic model for diabetic angiopathy [99]. Thus, CAM of chick could be gainfully utilized for evaluating effects of high glucose on vascular endothelial cells and on angiogenesis *in ovo*.

Conclusions

The chick can serve as an alternative to mammalian models for the study of specific aspects of diabetes. Isolated islets from chick pancreas offer a simple, economical, and effective system for screening for antidiabetic agents and possibly for treating poultry diabetes in case one is needed in future.

Chick CAM can be employed to detect hyperglycemia-induced abnormalities in angiogenesis. Developing chick embryo with a shell-less culture system can provide a suitable alternative for investigating high glucose-induced secondary complications of diabetes, drug testing, and analyzing developmental malformations, thus sparing pregnant mammals from being used for this experimental purpose.

With the establishment of biotechnology courses for graduate and postgraduate level students, the chick would provide an excellent noncontroversial, non-seasonal, and inexpensive alternative model system to analyze specific aspects of diabetes.

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