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Chicken embryo: a useful animal model for drug testing?

L.N.M. RIBEIRO[1], A.E. SCHLEMPER[2], M.V. DA SILVA[3], B.B. FONSECA[1,2]

[1]Institute of Biotechnology, Federal University of Uberlândia, Uberlândia, Brazil
[2]School of Veterinary Medicine, Federal University of Uberlândia, Uberlândia, Brazil
[3]Central Animal Facility of Federal University of Uberlândia, Uberlândia, Brazil

Abstract. – The use of chicken embryos (CEs) as an *in vivo* experimental model for different pharmaceutical purposes is not a novelty. However, in recent years, the number of reports employing CE to evaluate several parameters, such as the toxicity and efficacy of drugs and/or nanosystems, has increased. Therefore, this review discusses the relevance of CE for drug testing, emphasizing the inoculation routes and the embryonic stages. The challenges to be overcome, as well as some practical recommendations to allow CE to be more explored as a promising *in vivo* model in drug analyses, are also highlighted.

Key Words:
Allantoic fluid, Amniotic fluid, Chicken embryo model, Drug delivery system, Shell membrane, Toxicity, Efficacy.

Introduction

The scientific community has been investigating alternatives with lower costs and fewer ethical and logistical problems for the performance of analytical *in vivo* assays. In this sense, the chicken embryo (CE) has become attractive. This type of model has been applied to evaluate several parameters and is useful in angiogenesis, ischemia, cancer treatment, drug delivery and (nano)toxicity tests[1-4].

In vivo assays refer to the experiments conducted with or within an entire living organism, that is, assays through animal models, which are essential for preclinical tests[5]. CE is accepted as an animal model for different purposes[6,7]. Specifically, in drug testing, CE with approximately 7 embryo incubation days (EID) should be used for the analyses of teratogenesis and toxicity[8]. Moreover, due to more developed and mature organs, the use of CE in the advanced (>12 EID) embryonic stages can be useful to determine the release profile and toxicity of drugs[9].

The advantages of CE are largely reported in comparison with traditional mammal models, such as its low cost and reduced maturation time. Taking into account the rapid CE development stage, drug absorption, distribution, metabolism and excretion, specifically related to the route of inoculation and drug tested, must be deeply explored. This manuscript focuses on the use of CE in toxicity and efficacy tests of free molecules and drug delivery systems (DDS). Additionally, relevant information on the experimental protocols was provided to increase the reliability of the results of drug testing. This versatile alternative model still must be systematically explored, considering some parameters in the experimental design, such as inoculation routes, different stages of embryo incubation, and CE physiology, to contribute to the acquisition of robust data from these assays and enable dissemination of the method.

CE Anatomy and Physiology of the Central Tissues According to Embryonic Development Stages

The Development of the CE Is Fast

The development of CE is rapid, corresponding to a total of 21 days[9] (Figure 1). In the first 24 hours of embryonic development, important events occur in CE formation. The first blood vessels are extra-embryonic and become visible in the yolk sac (YS) from 23-24 incubation hours (IH). The first heart beats can be detected at 33-38 IH, and the circulation has become well established at approximately 51-56 IH[9]. Generally, the allantois starts to develop at 3 EID, while the embryo is surrounded by the amnion in such a period[9,10]. Allantois fusions with the chorion to form the chorioallantoic membrane (CAM) are completely visible at 6-7 EID.

CAM is located in close contact with SM (Figure 1), promoting gas exchange[11]. The amnion

Corresponding Author: Belchiolina Beatriz Fonseca, MD; e-mail: biafonseca@ufu.br

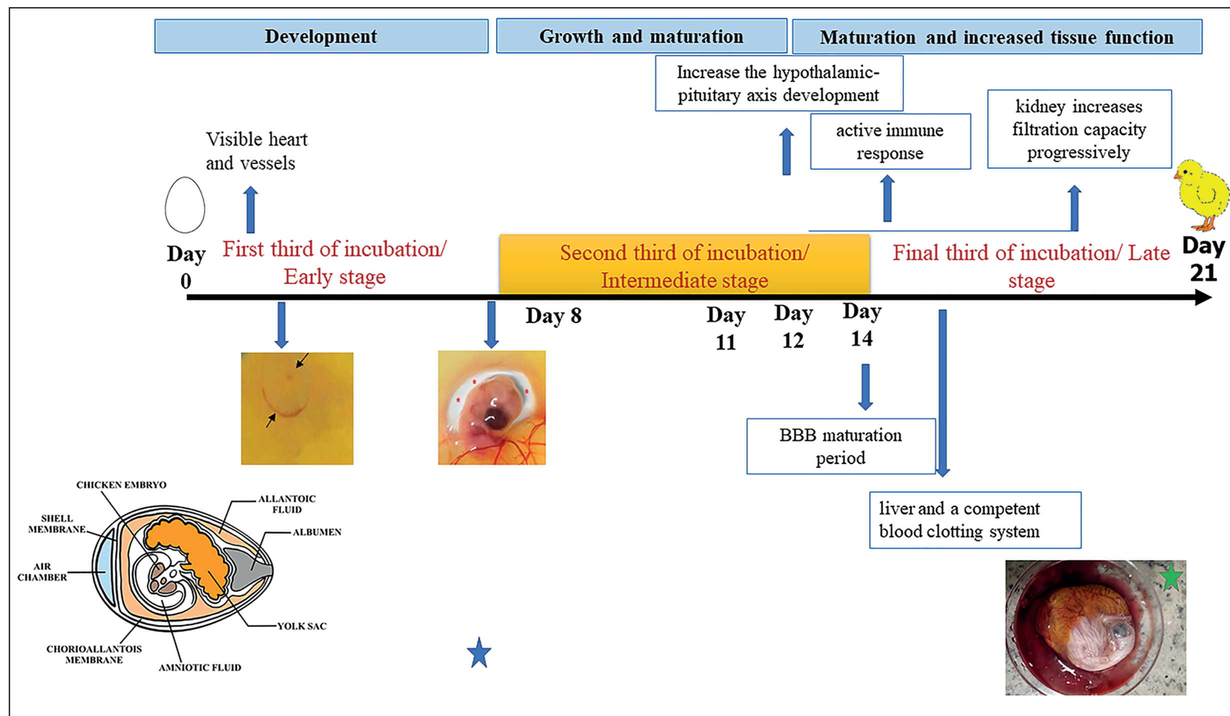

Figure 1. Chronological scheme of the chicken embryo (CE) development stages until 21 EID. The vessels and heart become visible from 24 IH (black arrow). At 7 EID, central tissues are formed, and embryonic annexes can be seen. The red star shows the amnion. The blue star indicates the anatomy of the CE and its embryonic annexes. At 8-11 EID, the tissues are in the growth and maturation phases. At 12 EID, the organs increase their functions gradually. The green star shows a digital photograph of a fully developed CE at 16 EID. This photo is a personal file from a project approved by the Ethics Committee of the Federal University of Uberlândia (Certificate 008/21).

and YS have the primary functions of nourishing the CE[12]. An important function of allantoic fluid is allowing the deposition of excreta[13]. After 7 EIDs, the embryo already has a nervous system, and it begins to perceive pain[14].

Embryonic development can be divided into 3 stages: the first third of the incubation or early stage (up to 7 EID), the second third of the incubation or intermediate stage (in the range of 8-14 EID) and the final third of the incubation or late stage (from 15 to 21 EID) (Moran[12], 2007) (Figure 1). However, if the formation, maturation and organ development periods are considered, many tissues start to exert their functions from 11-12 EID. At 7-10 EID, the main organs are formed but still need to go through growth and maturation[9,15-17] (Figure 1). From 12-14 EID, the tissues undergo considerable maturation. At 12 EID, there is an increase in the main hypothalamic-pituitary axis development[18].

The blood-brain barrier (BBB) has reduced permeability of the vascular walls of neural vessels from 14 EID, when the BBB is becoming mature[17]. The immune system has an active immune response at approximately 15 EID[19,20]. The kidney increases the filtration capacity progressively from 12-18 EID[21]. At 16 EID, the CE has a functional liver and a competent blood clotting system[22]. CE is composed of a wide variety of proteins and enzymes involved in metabolism, which exhibit intense activity at 16 EID[22,23]. In the last incubation phase (from 14 EID), CE passes into the amniotic cavity. CE digests this mixture orally, being absorbed into the small intestine and accumulating glycogen reserves in muscle and liver tissues[12] (Figure 1).

In ovo Experiments: the Role of the Different Inoculation Routes in Chicken Embryos

The main routes employed in *in ovo* experiments are *via* yolk, albumin, CAM, allantoic fluid (AlF), shell membrane (SM), amniotic fluid (AmF) and YS of CE.

The sample inoculation in the top of the egg reaches SM through the air chamber (AC)[24-27] using

Table I. Main uses of chicken embryos as experimental models.

Goals	Target	Route	Age at inoculation	Reference
Ophthalmology	Retinal surgery	CAM	12 and 18 EID	87
	Retinal regeneration	CE	4 EID	88-90
Angiogenesis	Vessels of CAM	CAM	1-5 EID	91
			9 EID	38
Ischaemia-Reperfusion	Vessels of YS	YS	3 EID	92
Skin	Irritation and/or toxicity	CAM	After 9 EID	40
	Grafting		7 EID	41
Liver	Grafting	CAM	9 EID	42
Regeneration of living bone	Grafting	CAM	10 EID	1
Spontaneous metastasis	Grafting	CAM	10 EID	37
Tumour	Grafting	CAM	9 EID	45
			8 EID	76
			8 EID	44
			8 EID	43
Type I Diabetes	Drugs in the amnion	AmF	8 EID	46
Type 2 diabetes mellitus	Insulin-mimetic compounds	SM	11 EID	47
Endometriosis	Grafting	CAM	CAM (in the sharp end of the shell). Inoculation at 6 EID	48
	Grafting		10 EID	49
Drug	Toxicity	Yolk	4 or 8 EID	25
		YS	4 EID	79,80
		Albumin	before incubation, 0 EID	78
		SM	10 EID	24-27
		CAM	10 EID	68
Nanosystems	Toxicity	SM	3 EID	71
	Toxicity	CE	3EID	72
	Angiogenic activity	CAM	10 EID	75
	Angiogenic activity	CAM	5 EID	77
	Antioxidant property	IV	15 EID	65

an injection needle (2 cm long and external diameter of 0.5 mm)[25]. SM is formed by a porous lipid bilayer. The internal and external layers have pores with sizes of 126 Å (or 12.6 nm) and 0.53 µm, respectively[28]. The pore diameters must be considered in the evaluation of drug permeation and release profiles from these barriers. Therefore, SM can be an attractive route for testing drugs with a molecular size smaller than 126 Å. However, the bioavailability of drugs on CE through this route has not yet been elucidated. This would be advantageous once there is a higher possibility of accessing the vessels without causing CE lesions during sample administration.

In fact, the constant movement of the CE within a fluid and its intense vascularisation[19] are challenges to successful sample inoculation *via* the AlF, YS and AmF routes. Thus, it is essential to differentiate embryonic deaths by experimental error in sample inoculation from those occurring as a result of the performed tests. The deaths registered within 24 hours of inoculation are usually considered nonspecific losses[29]. The access to the AlF route is relatively simple, requiring the use of a hypodermic 25 G needle with a 0.5 mm gauge. This route was successfully employed to replicate some microorganisms, such as viruses[30]. The inoculation procedure in the AmF route is not complex but deserves attention. Unpredictable events can occur, given the highly heterogeneous eggs. For instance, to successfully reach the amnion of CE at 18 EID, the hypodermic 22 G needle (0.7 mm gauge) was uniquely able to be used without inducing CE damage[31].

AlF, YS and AmF vias have been frequently explored for the administration of vaccines in the poultry industry[31,32]. Vaccine protection against viral challenges is dependent on the vaccine inoculation site. There is a decrease in the vaccinal viremia of such vaccines administered *via* AIF, with less success in protecting against the virus[32,33]. However, there are still no reports comparing the drug delivery and metabolism profiles dependent on the inoculation route in CE.

On the other hand, there are many descriptions related to access to CAM through different techniques[34] (Table I). For sample inoculation in CAM, it is recommended to use a CE with a minimal age of 8-9 EID, as younger CEs do not

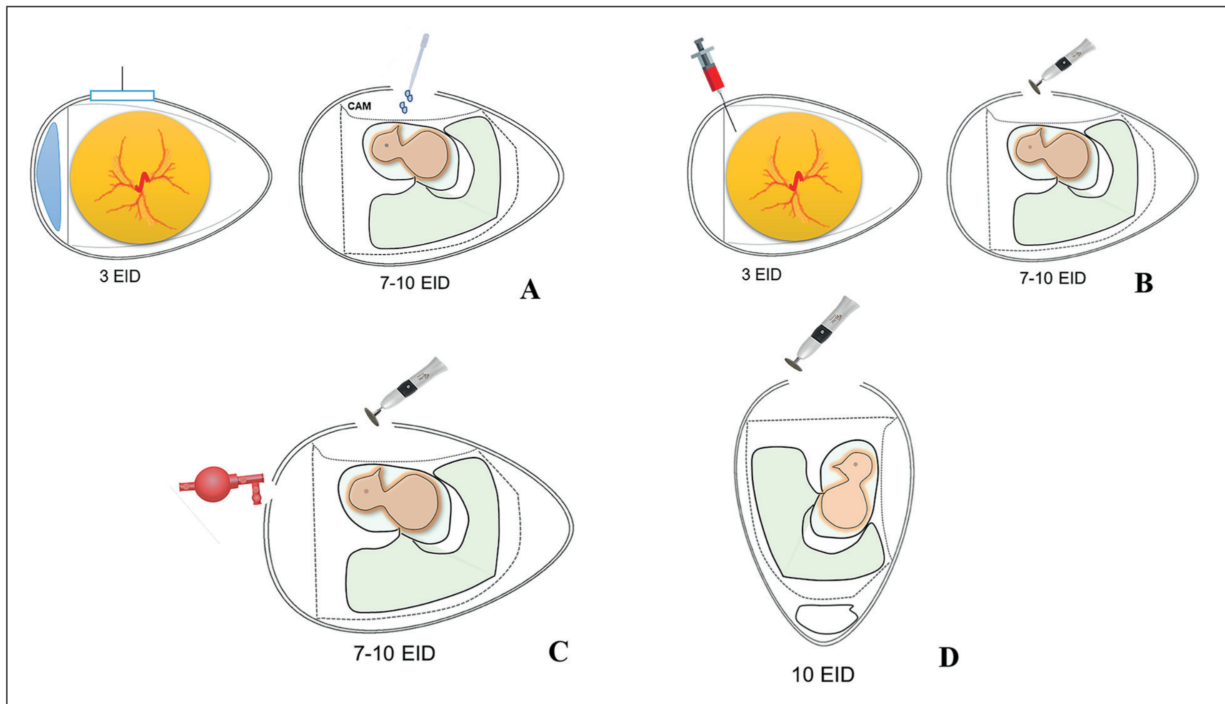

Figure 2. Illustrative scheme of the membrane reversal techniques, consisting of an incision and creation of a window, allowing better visualization of CAM. **A**, A window in the centre of the egg at 3 EID. CAM develops far from the SM, which can be accessed after 7 EID. **B**, Removal of the albumen at early stages of CE incubation. The albumen is removed at approximately 3 EID. Thus, CAM develops far from the SM, allowing its access from 7 EID. **C**, CAM reversion by pressure at the time of the experiment. An incision was made in the peel where AC was placed and the air was drawn. Then, CAM descended, and a window was created in the centre of the egg. **D**, Access *via* AC. A window was made at the top of the eggshell, where AC is located, and the membrane of the shell was gently removed, providing CAM access.

yet have totally developed vascular membranes[34]. CAM is vascularised and located close to SM, allowing oxygen exchanges through its pores[28]. It is responsible for calcium transport from the eggshell into the embryo, acid-base balance, and reabsorption of water and electrolytes from the allantoic cavity[11].

The successful administration of liquid samples in CAM should be achieved using CEs between 7-12 EID. This procedure is generally performed by SM scarification located in the middle of the egg without reaching the CAM. Then, a hole is made following the larger pole of the egg, and the membrane will naturally descend. Other *in ovo* techniques to access the CAM have been used in different experiments, as follows: SM incision at 3 EID[35], albumen removal at early stages of incubation[36] and reversion of CAM by pressure at the time of the experiment[37] or *via* AC[38] (Figure 2).

It is worth mentioning that an initial incision at 3 EID can change the pressure within the egg and prevent the connection between the SM and CAM[35]. Consequently, CAM can be accessed from 7 EID (Figure 2A). The partial removal of the albumen (Figure 2B) is a simple technique[36], but it can cause a reduction in the AlF and AmF volumes, decreasing the water content and the weights of both the residual yolk and CE body[39]. Another practical method is CAM reversion by pressure, which must be performed in CE from 7 EID (Crespo and Casar[37], 2016) (Figure 2C). However, such a method is dependent on operator capability and pressure equipment, as damage to the vessels can occur, resulting in CE bleeding and death. It is also possible to access the CAM directly *via* excision, opening a window in the AC and removing the SM carefully (Figure 2D)[38].

The Chicken Embryo Model as a Valuable Tool to Evaluate Drug Delivery Systems and Toxicity

The CE is an undoubtedly helpful tool for use as an experimental *in vivo* model for multiple applications, such as testing pharmacokinetics,

toxicity, grafting and regeneration of active molecules; targeting the skin, eyes, liver or bones; and targeting different routes (Table I)[40-42]. Additionally, this approach is currently used as a model for tumour treatments and angiogenesis[36,37,43-45]. The successful application of such a multipurpose model can be explained by the lack of immunocompetence of CE until approximately 14 EID[19,20], rich vascularity and the rapid speed of tumour growth in the CAM[2]. Type I[46] and type II diabetes[47] as well as endometriosis[48,49] are other successfully-induced diseases in this model.

The development of DDSs has been documented since the last century. In recent years, specialized DDSs have been widely described and are composed of different organic and/or inorganic materials or a combination thereof. The major goals are to minimize drug toxicity and prolong the drug release profile, resulting in increased bioavailability and efficacy. These systems can load different classes of drugs and active molecules, aiming at different purposes and administration routes. They are currently processed in colloidal, semisolid and solid pharmaceutical forms[50].

Unfortunately, despite the robust literature regarding the advantages of DDSs over traditional drug therapies, including *in vivo* and clinical trials describing exciting results, such systems often cannot reach the market and improve patient quality of life as designed[51]. In DDS development, there are some essential steps to ensure pharmaceutical product quality and efficacy to allow them to reach the market[52]. Several challenges still need to be overcome, such as the purity of excipients, product quality control, scale-up process, economic viability, biocompatibility and standardization of *in vivo* assays[53]. Specifically, two essential parameters deserve attention: toxicity and *in vivo* efficacy/activity assays.

Biocompatibility tests are currently performed to select the safest formulations to be submitted to efficacy assays and are usually conducted in mammal models. The safety of DDSs can be evaluated by several *in vitro* and *in vivo* assays, which is mostly ensured by *in vitro* cell viability tests in different cell lines[54]. However, these *in vitro* experiments can be performed through the reduction of tetrazolium salts (MTT) method. This method is currently employed to determine cell viability and proliferation and can present relevant limitations, especially in nanostructured DDS evaluation[55]. The reliability of results obtained through this method can be affected by several conditions, such as acid medium, pyruvate analogues and nanostructures[56-58], contributing to undervalued cytotoxicity determination by overrating cell viability[55]. Moreover, some drugs, such as chemotherapeutics, can interact with the MTT medium[59]. Additionally, some DDS structural properties, such as supramolecular arrangement, partition coefficient, surface charge, optical properties and/or catalytic activity, can affect the assay execution or detection apparatuses, resulting in misinterpreted data[60].

In this sense, alternative *in vivo* toxicity models can be employed as versatile approaches to determine DDS nanotoxicity, such as zebrafish, *C. elegans* and CE models[61-63]. The main advantages of such alternative models over traditional *in vitro* tests are the investigation of DDS toxicity in live tissues, cells and proteins of biological barriers of CE, as well as their interactions (Ribeiro and Fonseca[64] 2020). Additionally, this *in vivo* method provides robust toxicity results because it is not dependent on colorimetric analytical techniques to predict DDS toxicity (such results can be affected by environmental and experimental conditions); the affected organs can be studied and biochemically analysed; changes in some parameters of living animals, such as heart rate and spontaneous movements, can be monitored; and electronic microscopy techniques are currently used to elucidate morphological or functional abnormalities of target organs or individuals treated with DDS[64-68]. Therefore, *in vivo* toxicity models provide more consistent results that will direct the most desirable systems to further *in vivo* efficacy studies. An FDA guide (2006)[6] was provided, considering the use of the CE model as an alternative for the preclinical evaluation of pharmaceutical developments.

CAM and CAM-induced tumours are currently used to measure the (nano)toxicity and drug activity. Other relevant tools to ensure DDS safety are the analysis of embryo development and death rates, changes in the weights of embryos and their organs, and biochemical analysis of CE plasma and systemic/local side effects after DDS treatment[69]. Recently, the nanotoxicity of carbon nanoparticles assessed *in ovo* during CE embryogenesis was reviewed. The authors emphasized that the early stages of chicken embryogenesis are robust models for elucidating potential nanotoxicity effects on physiological maturation and oxidative stress[70]. In this sense, a nanotoxicity assay using the CE model elucidated the safety of the carbon nanoparticles. Different parameters, such as inflammation and apoptosis pathways, together with biochemical markers, were studied. It was observed that the

nanoparticles improved the levels of lipid peroxide and decreased the antioxidant and glutathione levels in the CE brain. Furthermore, some proinflammatory genes were upregulated, along with the relevant downregulation of apoptotic markers, such as caspase-8, caspase-3 and cytochrome c. The authors concluded that CE treatment with nanoparticles contributed to improved free radical production, followed by apoptotic responses[71]. Toman et al (2015)[72] described the development of alkylglyceryl-dextran-graft-poly(lactic acid) nanoparticles as a matrix for the brain delivery of several drugs. This system was structurally detailed, presented physicochemical stability and ensured safety through cell viability and *in ovo* nanotoxicity tests using 3-day CE. Such experiments were conducted by removing the vitelline membrane, followed by sample inoculation into the heart (near the aorta) of CE through a microneedle associated with a pumping device. After 24 h, the embryos were removed from the eggs and fixed for subsequent microscopic analysis. The authors ensured the safety of the formulation due to the absence of an acute toxicity effect[72].

The last step of DDS development before clinical trials may confirm the *in vivo* efficacy or therapeutic activity of the developed devices. The systems are submitted to specific *in vivo* efficacy tests to clarify the benefits of loaded molecules over controls (free or commercial active molecules). Such results will direct the samples to further human trials, the last step prior to approved use. Many DDSs have already been reported to study the efficacy of actives through the CE model[73], including liposomes, nanoemulsions, nanoparticles and hydrogels[74].

In recent years, many efficacy tests of different classes of drugs have been described using the CE model. Antiepileptic drugs administered to pregnant women currently increase the prevalence of congenital malformations in the foetus, such as heart and neural tube defects and facial clefts. Lacosamide (LCM) is a third-generation antiepileptic drug approved as an adjuvant therapy to treat seizures. Mete et al (2016)[67] evaluated the antiepileptic effect of LCM through a CE model in the early stages of chicken development. Thus, different concentrations of LCM were inoculated under the embryonic discs of CE. After 80 h, LCM-treated CE was evaluated by macroscopic and microscopic parameters. The authors noticed dose-dependent growth retardation and congenital malformations in all embryos treated with LCM. Unfortunately, this was not an expected result for a safe drug candidate[67].

Moreover, CE has widely been applied as an angiogenic *in vivo* model. Li et al (2019)[75] proposed using diaminopropane tetraiodothyroacetic acid (DAT) as an anticancer antagonist loaded by polymer nanoparticles. The *in vivo* angiogenic activity of free and loaded DATs was determined through a CE model. The samples were administered in CAM to measure the angiogenesis ability. The authors reported that even when used at lower concentrations, the polymer nanoparticles encapsulating DAT (50 ng/CAM) were able to inhibit angiogenesis at higher levels than free DAT (500 ng/CAM)[75]. Another work[65] investigated the antioxidant properties of redox nanoparticles (RNs) using CE. First, the authors induced intense oxidative stress in the CE prior to treatment with RN. They showed that RN was able to exert desirable protective effects, decreasing embryo lethality and suppressing lipid peroxidation in chicken serum[65].

Finally, CE was employed as an anticancer efficacy model. Such works that explored CAM as the inoculation route were recently revised by Victorelli et al[74] (2020). Recently, mesoporous silica nanoparticles functionalised by the folate antagonist methotrexate were inoculated in the CAM of thyroid tumour-induced CE, aiming for the sustained release of fingolimod. A decrease in thyroid-derived xenografts followed by an improved necrotic phenotype was observed when compared to CE tumours treated with the commercial drug[76]. Dias et al[77] (2018) developed polymer nanoparticles encapsulating imiquimod for skin cancer treatment. Thus, the samples were administered in CAM, and the antiangiogenic activity test was carried out. This experiment showed an antiangiogenic effect three times higher than that of the free drug, which was subsequently corroborated by stereomicroscope and histological images[77]. The versatility, viability, easy handling and robustness of CE have been confirmed to make this an effective analytical *in vivo* model to determine the toxicity and efficacy of active molecules[69].

Different Methodologies for Drug Testing

Several methodologies have been described for drug testing through CE. The heterogeneity of experimental conduction in similar assays is clear. For instance, several CE incubation times were used in analytical experiments testing drugs, with the sample inoculated in CE at 0[25,26], 1[78], 3[24],

4[79,80] and 10 EID[27,68]. Different routes have also been explored, such as yolk[25], YS[79,80], albumin[78], SM *via* AC[24-27] and CAM[68] (Table I). In addition, the samples were collected from CE at different times, with embryos at 6[80], 12[80], 14[24,25], 17[68], 18[80] and 20 EID[26,78,79] (Table I). In a toxicity assay, several parameters are assessed in a single model, such as embryo mortality, egg weight, embryos and annexes, oxidative stress, blood serum biochemical markers, amniotic and allantoic fluid biochemistry, red blood cell morphology, organ damage, deformed embryos and histopathological changes[24-27,68,79].

However, the current absence of an experimental design considering several parameters, such as the CE incubation, inoculation and sample collection times, and routes for sample administration, among others, can result in unreliable results. In the early stages of incubation, the embryo does not have immune, renal and hepatic systems, pancreatic enzymes, or fully formed intestine or BBB. Although most organs do not mature until a few days after hatching, in general, their activities are significantly increased after 12 EID and are very active at 18 EID[12,15-17,19-23,81-85]. These characteristics can generate different results from the same drug in embryos of different ages. Thus, death or embryonic damage in the early stages are caused by direct injury upon sample inoculation, without the contributions of the embryonic immune system or organs. Therefore, it is necessary to elucidate the most appropriate embryonic ages for each *in vivo* assay, considering the purpose of the analysis, specificities of the explored routes and the physicochemical properties of loaded drugs. Depending on these parameters, the absorption, distribution, metabolism and excretion of analysed molecules could be more analytically explored. The rational choice of the inoculation route and the stage of embryonic development will guarantee robust results, also allowing a decrease in the number of mammals in subsequent *in vivo* and clinical trial studies.

Ribeiro et al (2020)[68] described a pilot work determining the age and norfloxacin dose that did not kill the embryos and was also effective in the treatment of *Salmonella* Heidelberg (SH) infection. Such an approach is mandatory for drug pharmacokinetics, absorption, distribution, metabolism and excretion analytical assays. In an efficacy test, CEs at two different stages of development, 13 and 16 EID, were inoculated (*via* AIF) with valproic acid and lamotrigine, targeting the CE brain (still in formation). Both drugs successfully reached the brain. However, the drug concentration in the CE brain was higher when injected at EID 13 compared to EID 16. This difference was attributed to the presence of a more mature BBB in older embryos[7]. On the other hand, younger embryos, in which their organs are in the maturation process or still absent, have served as essential tools in teratogenesis or preliminary drug toxicity tests. In pharmacokinetic and systemic toxicity tests, older CEs may be preferred, as they have more mature organs. Nevertheless, according to the CE physiology at different incubation times, the analyses of drug release profiles and metabolites may be conducted differently.

In CE, AIF is in contact with CAM[11]. Therefore, the drugs orally administered by this route can be diluted in the fluid. The diluted drugs can reach CAM or YS vessels, passing to the embryos. The systems that aim for intraoral application can be tested by CE through different routes. Sample inoculation *via* AmF can also be useful for simulating intraoral administration. The CE will subsequently digest, absorb and digest the samples[12]. In addition, YS binds directly in the CE intestine, and the yolk is absorbed as the embryo grows[39]. Therefore, YS is probably the ideal route for testing drugs with intestinal absorption. The high number of blood vessels in CAM[86] facilitates the access of drugs directly into the animal bloodstream, which is essential for parenteral drug testing.

Perspectives

This review emphasized understanding whether some experimental parameters of assays (focused on drug testing) conducted through the CE model will provide reliable results. The choices of CE incubation age, inoculation route and experiment duration (or sample collection times) must be rationally planned and performed to modulate the analysed responses. The experimental design also should consider the physicochemical properties of drugs or DDS.

The embryonic stage is an essential parameter to be considered in the experimental design due to the time-dependent organ maturation in embryos. CE with at least 11-12 EID may be preferred to conduct active experiments on pharmacokinetics, pharmacodynamics and/or systemic toxicity. At this point, the central organs (liver, kidney, circulatory, immune system) are more mature, providing robust results. The CE can also be employed

for preclinical assays. On the other hand, younger embryos can be useful in toxicity drug testing, being similar to assays conducted through cell lines, with the advantage of having multiple tissues formed (although not yet functional). CE is also a versatile experimental model for teratogenesis and genotoxicity analyses.

Once a rational CE age selection is performed while also considering the experimental purpose, the next step is the selection of the best route for sample inoculation. This decision should be driven by simulating the site of administration of tested drugs/DDS. There is still a lack of data correlating the inoculation route with the different administration routes (parenteral, intraoral, topical) in mammals, except for CAM. CAM is the most studied inoculation route, being explored to simulate parenteral, intraoral and topical administration with success. Specifically, regarding intraoral application, AMF is clearly the preferred route, and YS is the best choice for intestinal mucoadhesive drugs and DDS evaluation.

It is worth mentioning that the selection of proper hypodermic needles to access the inoculation route is also mandatory to prevent CE damage and early death. Additionally, the CE deaths after 24 hours of sample inoculation should be discarded from the analysis, as losses could result from human experimental error. In addition, considering the lower risk of deaths or injuries caused by sample inoculation in CE, together with the easy handling procedure, SM should be the preferred route for drugs with molecular sizes smaller than the pores or with a high partition coefficient, allowing direct sample contact with CAM.

Last but not least, after a rational experimental design, other approaches can be further explored in the CE model. In drug development, there is a huge demand for specific toxicity and efficacy *in vivo* and preclinical assays to approve new drugs. CE is certainly a promising model to evaluate cardiovascular toxicity, anaesthetic and opioid effects, wound healing, bacterial and cancer treatments, among others.

Conclusions

The CE is a multifaceted *in vivo* experimental model providing analytical data for several approaches in drug testing. Many reports have focused on drug toxicity and efficacy through this model, proving that CE is an excellent alternative model for such purposes. The available works suggest that experimental planning using CE as a biological model must focus on embryonic age, route of inoculation, and adequate instrumental and drug physicochemical properties to obtain reliable drug toxicity and efficacy data. This review attempted to clarify the correlations of CE physiology, inoculation and intended administration routes for drug and DDS testing. It was also an attempt to spread this versatile and still poorly studied alternative model.

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Author Contributions

Belchiolina Beatriz Fonseca realized the need to write the article and invited the other participants. Belchiolina Beatriz Fonseca, Murilo Vieira da Silva and André Eduardo Schlemper wrote the sections about chicken embryo anatomy, physiology and the roles of the different inoculation routes in the chicken embryos. Ligia Nunes de Morais Ribeiro and Belchiolina Beatriz Fonseca wrote sections about chicken embryos as a valuable model to evaluate drug delivery systems and toxicity and different methodologies for drug testing. Belchiolina Beatriz Fonseca and Ligia wrote the introduction, perspective and abstract. Belchiolina Beatriz Fonseca, Ligia Nunes de Morais Ribeiro, Murilo Vieira da Silva and André Eduardo Schlemper made the figures and tables. Belchiolina Beatriz Fonseca and Ligia Nunes de Morais Ribeiro edited the manuscript. All authors reviewed and contributed to the improvement of the entire work.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

ORCID ID

Lígia Nunes de Morais Ribeiro: https://orcid.org/0000-0001-6097-5449
André Eduardo Schlemper: https://orcid.org/0000-0002-0841-9485
Murilo Vieira da Silva: https://orcid.org/0000-0002-3397-0113
Belchiolina Beatriz Fonseca: https://orcid.org/0000-0001-8485-078X

Data Availability Statement

All data generated or analysed during this study are included in this published article.

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