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The Chicken Embryo: An Alternative Animal Model in Development, Disease and Pharmacological Treatment

Oykum Kaplan-Arabaci[1] | Zuzana Dančišinová[1,2] | Ragnhild Elisabeth Paulsen[1]

[1]Section for Pharmacology and Pharmaceutical Biosciences, Department of Pharmacy, Faculty of Mathematics and Natural Sciences, University of Oslo, Oslo, Norway | [2]Institute of Neurobiology, Biomedical Research Center, Slovak Academy of Sciences, Košice, Slovakia

Correspondence: Oykum Kaplan-Arabaci (o.k.arabaci@farmasi.uio.no) | Ragnhild Elisabeth Paulsen (r.e.paulsen@farmasi.uio.no)

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ABSTRACT

To examine various medications and substances, in vivo models such as rats and mice are routinely used. However, it is utterly desirable to reduce extensive amounts of animals for these experimental models, which are costly and time-consuming. Animals are frequently put through a variety of procedures that could cause them pain, distress, or even harm; therefore, it is important to think about the ethical ramifications of using them in research. Thus, by following the three R's of animal research: reduction, replacement, and refinement, living animals used in studies should be minimized. The embryo of *Gallus gallus*, the domestic chicken, is a great model to research many different diseases and conditions. Its efficient blood supply from the chorioallantoic membrane gives us a unique possibility to administer chemicals or cells to the embryo in a noninvasive manner. In this review, we evaluate some advantages and disadvantages of using the developing chicken as an alternative in vivo model for development, disease, and pharmacological treatment. We focus on the top two leading causes of death: neurological disorders and cancer. We present a number of studies that describe the use of the chicken embryo in neuroscience and neurodevelopment research, in cancer research, and pharmacodynamic and pharmacokinetic studies. These studies show that the chicken embryo is an inexpensive, readily available, self-sufficient model with a short incubation period, high accessibility, and ideal for drug screening, making it an appealing model that can provide insightful biological and pharmacological information.

1 | Introduction

Laboratory animals such as mice or rats are used as in vivo models to study drugs and chemicals. These models help to understand their mechanisms of action as well as toxicity after direct or indirect exposure. However, toxicity evaluation using these animal models often requires a large number of animals, which is money- and time-consuming [1]. When conducting animal research, it is also essential to consider the ethical implications of using animals in experiments, as they are often subjected to various procedures that may involve pain, distress, or even harm. Therefore, researchers working in the biosciences field and who use live animals in research must try to minimize the impact of animal research by following the principles of the three R's: reduction, replacement, and refinement [2]. Using nonanimal models and techniques, such as cell lines, tissue samples, and alternative organisms such as bacteria, is generally considered to be significantly cheaper and faster and essentially prevents the ethical concerns typical for animal models [3]. However, these models often encounter certain limitations, such as the absence of data on the metabolic response of animals and also the lack of information on the consequences of long-term exposure to humans [4].

Abbreviations: APP, β-amyloid precursor protein; Aβ, amyloid beta; BBB, blood brain barrier; CAM, chorioallantoic membrane; CNS, central nervous system; EID, embryo incubation day; HH stage, Hamburger and Hamiltonian stage; I/R, ischemia–reperfusion; IH, hours of incubation; MAP 2, microtubule-associated protein 2; NGF, nerve growth factor; NMDA, N-methyl-D-aspartate; PAX6, paired box 6; POPs, persistent organic pollutants.

Oykum Kaplan-Arabaci and Zuzana Dančišinová contributed equally to this work.

The chicken embryo, fetus, and/or blood supply represent an alternative model for nonclinical safety studies that respects the 3R principles and serves as a more ethical choice for experimental purposes. Other advantages include the fact that the development of chicken embryos is similar to that of mammals; they are cheap, readily available, and have a short incubation time. There is full control of the number of embryos as well as the developmental stage, which allows for rapid research [5]. The laying hen produces a number of embryos at once with a similar genetic background and a robust microbial community (Lee et al. 2019) [6], but which are independent of maternal influences on neuroembryogenesis and gut microbiota development. The chicken embryo is therefore an important model for safety evaluation in maternal-fetal medicine and mechanistic studies due to its biological properties, such as high reproducibility, autonomous development, statistical independence between individual embryos, accurate litter size, availability, and easy in vivo experimental manipulation compared to humans and rodents [7]. Chicken embryos are ideal models for evaluating the impact of chemicals and biomaterials, focusing mainly on their teratogenic or embryotoxic properties [8].

Neurological disorders are the second greatest cause of death globally and the major cause of disability [9]. As the world's population ages and expands, more increases are predicted. This increase in the total number of affected individuals shows that further improvements in the diagnosis, treatment, and prevention of severe neurological illnesses are needed. Therefore, immediate action is required to reduce this burden, and for this purpose, there is an urgent need for reliable models to study neurological disorders. Cancer is an epidemic disease that is the leading cause of death worldwide [10]. The absence of a complete understanding of cancer biology is a key limitation to research and the development of new treatment strategies, as well as the very understanding of cancer cell invasion and metastasis. The models used to identify new approaches to cancer treatment are constantly changing and expanding toward advanced preclinical studies. Although none of the cancer models are considered ideal, as each is associated with fundamental limitations that limit its application, bridging the gap between preliminary cancer research and translational medicine. The present review summarizes the potential and limitations of the developing chicken as a promising alternative in vivo model in development and disease, specifically focusing on neurological disorders and cancer, and their pharmacological treatment. The literature used in this review for the neurodevelopment and neurological disorders part is exhaustively selected and includes all the studies done in chicken; and for the cancer part, it was selected based on a detailed search for the most recent research and studies, especially those not already used in other review articles [11, 12].

2 | Chicken Embryo Model and Its Developmental Stages

Aristotle first began dissecting chicken embryos in approximately 350 BCE, and since then, the chicken model system has significantly contributed to the foundation for our understanding of human development [13]. The chicken embryo has several characteristics that make it a desirable animal model: it is phylogenetically closer to mammals than other alternative models such as zebrafish and nematode worms. It has well-developed and characterized CNS, cardiovascular, as well as respiratory systems. It is also conveniently sized, requires a short incubation period of 21 days, and is easily accessible during both the *in ovo* and *ex ovo* stages of incubation. Development is easily followed, which is useful for microsurgical and imaging operations. Moreover, each egg is independent and able to survive on its own and grows into an embryo at 37°C–39°C and 45%–55% air humidity without the need for expensive facilities or equipment [13, 14].

Due to the growing interest in using the chicken embryo as an alternative model organism, detailed descriptions of the overall development and the formation of individual organs and systems of the chicken embryo have been available. Viktor Hamburger and Howard Hamilton (1951) published a study describing the entire period of chick development from the first cell divisions to hatching. The development of the chicken embryo is generally a rapid process and corresponds to a total of 21 embryo incubation days (EID) [14] (Figure 1). The authors of the study defined three main stages of development, which retained the designation Hamburger and Hamiltonian (HH) stages of chicken development, which is still commonly used today. Early (up to 7 EID), middle or intermediate (between 8 and 14 EID), and late stage (from 15 to 21 EID) are distinguished [15]. During the first two stages, significant events occur, such as the formation of individual organs and systems, followed by the growth and maturation of entire organ systems (around 12–14 EID) [14]. Already after the first 23–24 h of incubation (IH), the first extraembryonic blood vessels form and become visible in the yolk sac. The first heartbeats can be detected around 33–38 IH, and the presence of all vessels and steady circulation is clearly visible around 51–56 IH. The allantois begins to develop at 3 EID, while the embryo is surrounded by the amnion. The fusion of the allantois with the chorion to form the chorioallantoic membrane (CAM) is fully visible at 6–7 EID [16]. Although the first sensory afferent nerve fibers develop at 4 EID, closure of multisynaptic reflex arcs does not occur until 7 EID [17]. Nociception in the chicken embryo is shown at 13 EID by electroencephalic recordings (EEG) [16, 18]. This stage could serve as the foundation for a future legal restriction that is more stringent regarding the use of chicken embryos during the final third of development. Since self-reporting, which is the gold standard for pain detection in humans, is not possible in animals, current studies use indirect methods such as changes in heart rate and blood pressure [19]. Weiss et al. investigated changes in blood pressure and heart rate in response to a noxious mechanical stimulus at the base of the beak in chick embryos between 7 EID and 18 EID. Significant changes in blood pressure and heart rate were detected at 16 EID to 18 EID [20]. At 9 EID, the embryo starts to look like a bird, and the opening of the mouth also appears. At 12 EID, there is an increase in the development of the main hypothalamic–pituitary axis, and the first few feathers are visible. The blood–brain barrier (BBB), one of the most important elements for neuroscience studies, matures from 14 EID [21], sharing great similarities with the human BBB [22, 23], and an active immune system

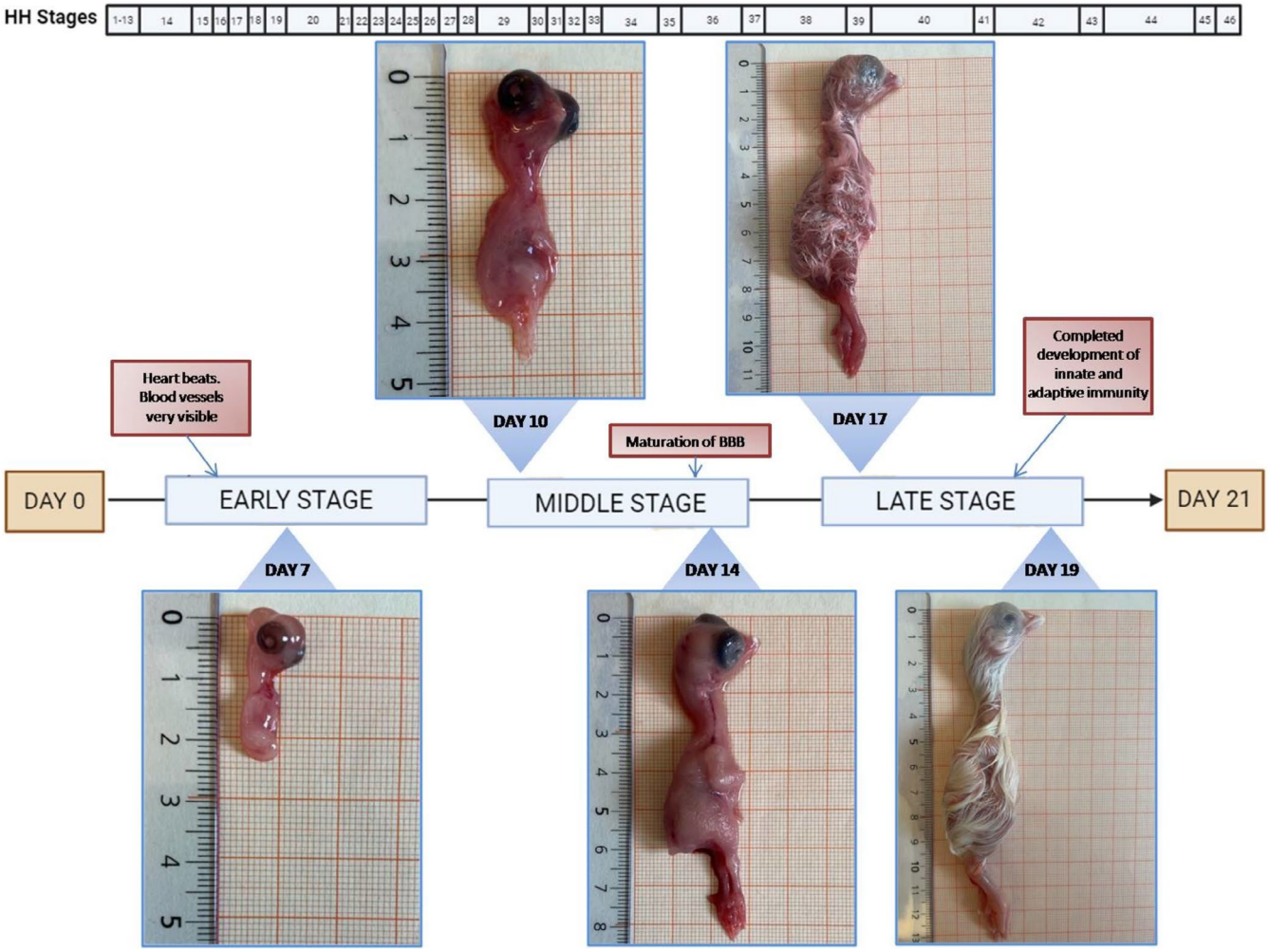

FIGURE 1 | The development of the chicken embryo during the 21 embryo incubation days (EID) in comparison to Hamburger and Hamiltonian (HH) stages. Early (up to 7 EID), middle or intermediate (8–14 EID), and late stage (15–21 EID) are marked. The first extraembryonic blood arteries grow and become evident in the yolk sac as early as the first 23–24 h of incubation (IH). Around 33–38 IH, the first heartbeats can be observed, and between 51 and 56 IH, it is evident that all of the vessels are present and that circulation is steady. At 3 EID, the embryo is covered in amnion, and the allantois starts to develop. At 6–7 EID, the chorioallantoic membrane (CAM), which is formed by the fusion of the allantois and chorion, is fully visible. The major hypothalamic–pituitary axis is developing further at 12 EID, and the first few feathers are beginning to show. From 14 EID, the blood–brain barrier (BBB) reaches maturity. Starting on 14 EID, the final incubation period, there is a loss of amniotic fluid, followed by the retraction of the yolk sac into the body cavity. BBB: Blood brain barrier.

responds from approximately 15 EID [24]. In the last incubation phase (from 14 EID) there is a loss of amniotic fluid. On 18 IH, the growth of the embryo is almost complete, and the yolk sac remains outside the embryo. The head is under the right wing. On 19 IH, the yolk sac retracts into the body cavity, and the embryo occupies most of the space in the egg. Later, the yolk sac completely retracts into the body, and the embryo becomes a cub (breathes air through the lungs), and internal and external pipping occurs (Figure 1).

The growth and development of chicken embryos are dependent on minerals, lipids, carbohydrates, and essential amino acids that are present in the eggs [25, 26]. The egg shell, which mostly consists of calcium salts, is the main stock of minerals. In addition, it protects the embryo and is porous to allow oxygen to the embryo [27, 28]. Egg yolk is the primary source of nutrients for developing embryos and have an impact on their viability [29]. It has been observed that the egg white, also named albumen, is essential for the initiation of embryonic development because it supplies water as well as nutrients to the developing embryo and acts as a barrier against bacterial invasion [30]. The proportion of these components change by time and reaches a point at which the embryo, egg yolk, and egg shell occupy the majority of the egg (Figure 2).

3 | Chicken Embryo and Its Use in Neurodevelopment and Neurological Disorders

The chicken embryo, a prominent model in developmental biology, has been essential to the understanding of neurodevelopment [31]. Wilhelm His made the famous discovery of the neural crest in the chicken embryo in 1868 [32]. Rita Levi-Montalcini, who also successfully researched chicken embryos, was awarded a share of the 1986 Nobel Prize in Medicine or Physiology for her discovery of nerve growth factor (NGF) [33, 34].

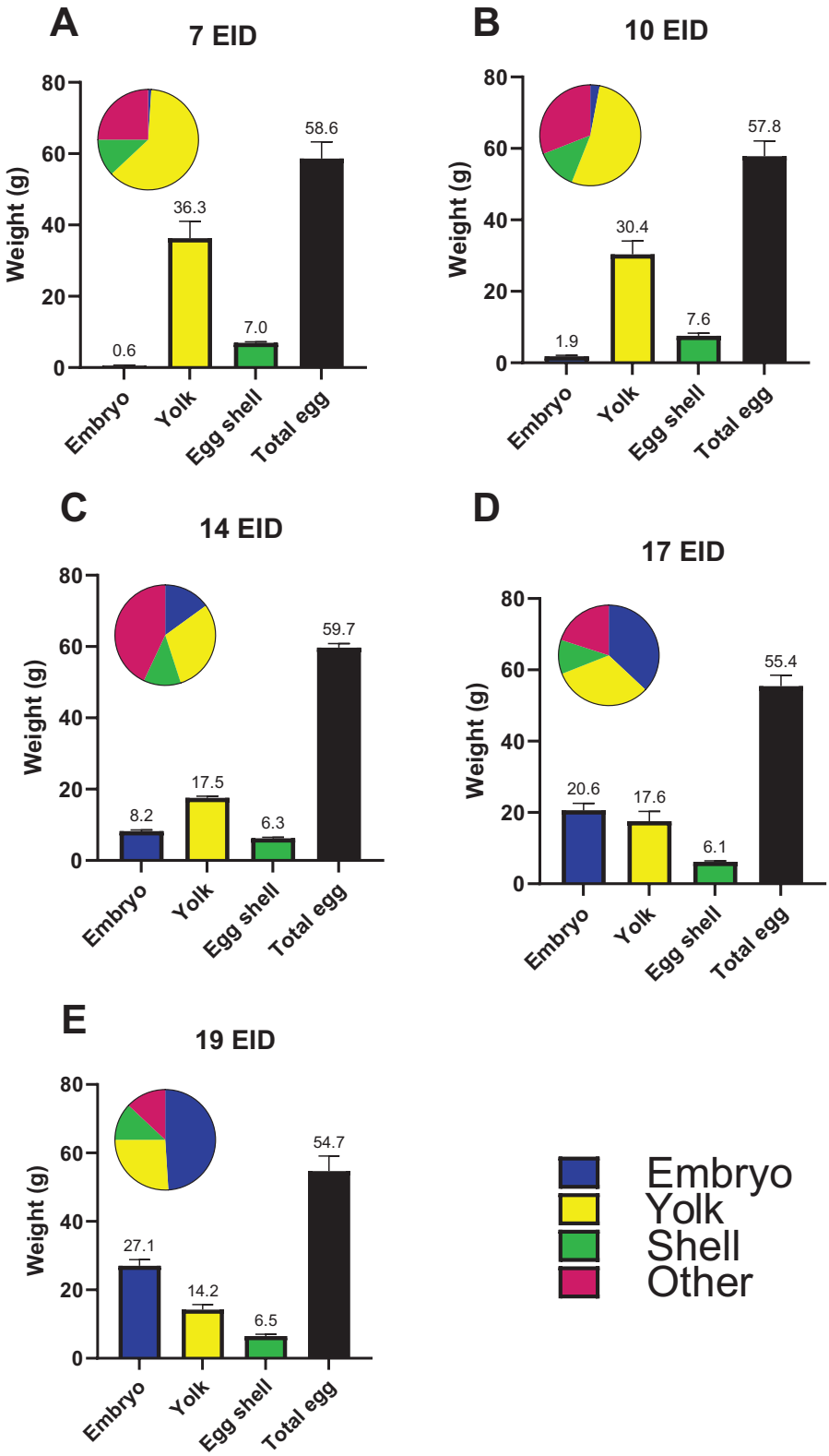

FIGURE 2 | Content of the chicken egg at different embryonic stages. Fertilized eggs (*Gallus gallus*) were received from Nortura Samvirkekylling (Våler, Norway) and incubated at 37.5°C in 45% relative humidity in an OvaEasy 380 Advance EXII Incubator (Brinsea, Weston-super-Mare, UK). Please note the weight increase of embryo and decrease of yolk. Other components, shown in pink include egg white (not observed at all at 19 EID), allantois, and amniotic fluid. It is also interesting to see a transient increase of other components up to 14 EID, probably due to the increase of amniotic fluid, and it starts decreasing thereafter. $n = 5$ for 7, 10 and 14 EID; $n = 8$ for EID 17; $n = 4$ for EID 19. Data were presented as mean ± standard error of the mean (SEM). EID: Embryo incubation day.

Cerebral cortex is the major area of the brain, playing an important role in learning and memory, emotions, and consciousness. However, human cerebral cortex consists of 6 layers including different types of neurons and has differences in many aspects, which makes it difficult to model in other organisms. The cerebellar cortex on the other hand, is a major model system for research on chicken brain development, due to the highly conserved structure between chicken and human [35]. When Ramon y Cajal initially characterized the anatomy of the cerebellar cortex in 1911, he said that it might be considered a "law of biology" because of how conserved it was across species [36]. In terms of modeling the broad characteristics of CNS development, the cerebellum is considered to be a suitable fit. Numerous, easily accessible, and capable of being purified and cultured in vitro, cerebellar granule neurons are the most homogenous neuron population in the brain. They go through every important step of brain development, making them the ideal model for researching the processes behind cell proliferation, differentiation, migration, and death [37, 38]. The migration of granule neurons through the layers of the cerebellar cortex, from the external granular layer to the internal granular layer, is a crucial aspect of cerebellar development and occurs mainly after 17 EID. This migration mostly happens in certain mammals, like humans, after birth [39].

Since the structure of the cerebellar cortex is highly conserved across species, as noted by Ramon y Cajal, histologic analysis of changes in the cortex is pertinent to a comparable environment in humans. Some distinctions do exist. Only mammalian species have the vermis, a medial enlargement of the cerebellum, and the timing of granule neuron migration varies throughout species, which must be considered when designing experiments. By the 25th week of gestation, the external granular layer of the human embryo reaches its maximum thickness, and the process of migration persists long into the first year of life [40, 41]. Cerebellar granule neurons migrate in the chicken brain prenatally, as opposed to the scenario in the rodent cerebellum, where granule neurons migrate between postnatal days 4 and 15, and the external granular layer forms postnatally [42]. Granule neuron migration in fish either does not occur at all or follows a different pattern from that of chicken and mammals [43]. Another benefit of using the chicken embryo model to research injuries to cerebellar development is that granule neuron migration in the chicken occurs at a fetal period. This has been applied to evaluate the effects of glucocorticoids on neurodevelopment [44, 45], as glucocorticoid exposure to the brain is a clinically significant concern for the newborn [46]. The developmental problems brought on by bisphenol A has been studied using the same model [47], and the outcomes are consistent with those found in a mouse model [48]. Disruptions in the expression of Paired box 6 (PAX6), a transcription factor that is crucial for cerebellar migration and development has been associated with autism [49, 50]. As a calcium-binding protein that is highly expressed in the cerebellum of different species, calbindin D28k has been studied in chickens [51–53], and it has been linked to autism [54] and hereditary ataxia [55] in humans. Microtubule associated protein 2 (MAP2) has been examined in chickens [56, 57], has involvement in neuronal growth and plasticity [58], and has been demonstrated to be relevant in human traumatic brain injury [59] and Rett syndrome [60]. These are some examples of indicators whose outcomes are applicable to human conditions and which could be used as targets in safety studies.

It is noteworthy to mention that the effects of the gut microbiome on particular biochemical pathways, general physiology, the nervous system, and behavior are becoming more and more well-established [61–63]. Because of its robust microbiota coming from the maternal oviduct and lack of reliance on maternal factors during development, chicken embryos may be used in experiments [64]. Huang and colleagues investigated the theory that too much tryptophan during pregnancy may impact a child's physical and mental development by altering the microbiota-gut-brain axis. The findings showed that exposure to tryptophan during embryonic development decreased the male offspring's body weight and aggression both before and during adolescence [65]. With more studies to come, chicken may thus be an emerging translational model for understanding fundamental mechanisms of alteration in the gut-microbiome-brain axis.

Several neuronal disease states have been modeled in the chicken embryo. Since the 1950s, chicken models have been used to examine a number of significant ocular conditions, such as glaucoma, corneal injuries, retinal detachment, and retinal degeneration. It has been the go-to vertebrate model for more than two millennia due to its quick evolution, ease of visualization, and accessibility for experimental manipulation [34]. Compared to most other regularly studied animals, chick eyes are notably larger, which is a significant advantage for surgical interventions as well as for collecting larger amounts of tissue for molecular studies and cell culture. Furthermore, embryonic chickens are a profitable model for regenerative eye biology because they have the ability to regenerate their retinas at specific stages [66].

One of the most widely investigated diseases, which is also affecting newborns, is epilepsy. For more than 40 years, researchers studying epilepsy have employed the homozygous Fayoumi strain of epileptic hens [67]. It has been shown that the electroencephalography of epileptic hens from EID 17 differs from that of normal chickens [68]. They have been applied to the assessment of anticonvulsant medications [69, 70], N-methyl-D-aspartate (NMDA) receptor antagonists [71], and magnetic resonance imaging [72]. Research has been done to look at how generalized seizures affect how the chicken brain develops and demonstrated a clear connection between alterations to the biophysical matrix of the growing chicken brain and recurrent seizures [73]. This study used noninvasive MRI techniques to identify alterations that were directly linked to seizure vulnerability, and the chicken is a great model for the therapeutic evaluation of pharmaceutical intervention due to its simple maturation period and accessibility to seizure frequency regulation.

Ischemia, which is defined as the lack of blood flow, deprives tissues and bodily parts of nutrients and oxygen. It is usually brought on by the restriction or blockage of blood vessels or arteries. In both humans and animals, reperfusion is the only treatment that consistently reduces the size of the infarct [74]. Fauzia and colleagues created an *in ovo* Hook model of

Ischemia–reperfusion (I/R) by blocking and releasing a chick embryo's right vitelline artery. When examining stress signals in stroke, this is a highly dependable and repeatable I/R model [75].

Interestingly, Carrodeguas et al. revealed that the chicken embryo possesses the necessary machinery to process β-amyloid precursor protein (APP) in both amyloidogenic and nonamyloidogenic ways, as well as to degrade amyloid beta (Aβ). Additionally, the sequence of the chicken APP gene is practically identical to that of the human sequence. Taking into account the fact that mice and rats are frequently employed as animal models for human diseases, despite the fact that their Aβ peptides differ in three of the forty-two amino acid residues, two of which affect the positively charged amino acid arginine, the chick embryo appears to be a natural model for Aβ research and possibly Alzheimer's disease [76]. There are plenty of studies focusing on learning and memory studies (For more details, please refer to the review articles [77–79]).

Last but not least, there are some more techniques used in chicken embryo, such as quail-chick chimeras, a labeling technique allowing tracing of certain cells and their progeny [80]; molecular genetic approaches such as in ovo electroporation [81] as well as transgenic approaches, which are described in detail in recent reviews [82, 83] highlighting the availability of the chicken animal model in neuroscience.

4 | The Fertilized Egg and Developing Chicken as a Cancer Model

At the beginning of the last century, the authors Murphy and Rous were the first to transplant primary tumor tissue onto a chicken embryo's highly vascularized chorioallantoic membrane (CAM). They thus demonstrated their rapid growth shortly after engraftment, based on which the *in ovo* model gained scientific interest as an alternative to the expensive, time-consuming in vivo mammalian model in preclinical oncological research [84]. Currently, the *in ovo* model serves as a valuable alternative to mammalian tumor models for investigating the characteristics of tumor growth, metastasis, angiogenesis, and consequently, the effectiveness of cancer therapies in preclinical oncology research.

The chicken CAM model has been shown to be a good experimental system for establishing a human patient-derived model. The CAM is a structure connecting the mesodermal layer of the allantois and chorionic membranes with a rich vascular network, which provides a unique biological microenvironment suitable for cancer cells (Ribatti 2016) [85]. The existence of a rich microvasculature inside the CAM system plays a vital role in the proliferation and survival of tumor cells for the CAM xenograft. The time course and onset of vascularization within the CAM tumor graft were clearly demonstrated and described in detail by Knighton et al. [86].

The development of innate and adaptive immunity of chicken embryos is completed at later stages, around 18 EID. Prior to this time, the chicken embryo is naturally in an immunodeficient state [87]. This immature immune system before 18 EID makes it an ideal environment for tumor engraftment and thus allows a higher rate of successful acceptance of tumor transplantation, either allograft or xenograft [87, 88]. Use of the chicken embryo model for immune-based studies was thoroughly reviewed in Garcia et al. (2021) [89].

Advantages of the CAM model are financial and time-saving, efficiency and high reproducibility, easy handling of chicken eggs, and the good visualization of the experiment. These features make the CAM model a suitable alternative to the most commonly used mouse model. As the CAM is covered with a rich vasculature, it is a suitable in vivo model, applicable for the assessment of angiogenesis and the investigation of the anticancer effects of various antiangiogenic agents [90].

A limiting factor for using the CAM model to study tumor angiogenesis is the alteration of the vasculature resulting from endothelial cell proliferation and neovascularization during embryonic development. Consequently, it is not easy to distinguish between tumor-associated neoangiogenesis and existing embryonic neovascularization. To avoid this problem, it is convenient to quantify vessels at a time when embryonic neovascularization is declining, which generally represents the period from 10/11 EID to 14/15 EID, when the endothelial mitotic index of CAM rapidly decreases after 11 EID [91] and the CAM structure at 13–14 EID expands with less complexity [92].

The CAM model can also be used to study tumor metastases. The spontaneous model allows the examination of metastases in the embryo after grafting tumor cells or samples obtained from the patient onto the surface of the CAM. Alternatively, the experimental model allows the study of metastases by inoculating tumor cells by direct injection into the allantoic vein [93].

This work demonstrates the great potential of the chicken model for use in cancer research studies; however, potential limitations of the model should always be critically considered.

One of the limitations of this model is the short observation period. Although rapid tumor development is considered one of the advantages of using this model in cancer research, the limited experimental time limits long-term observations and more time-consuming experiments, such as drug testing and remission studies. After the xenograft in 9 EID, there are only 9 days to treat and monitor tumor development *in ovo*, until 18 EID, due to respecting the regulatory rules on animal testing, which means that the experimental window is only 10 days. Thus, it is not possible to observe a potential recurrence of the disease, as is possible, for example, in a mouse model.

Studies in which the CAM model has been used to test different types of cancer cell lines or patient-derived tumors are summarized in Table 1.

5 | Chicken Embryo in Pharmacokinetic and Pharmacodynamic Studies

In the last years, pharmacokinetic (PK) and pharmacodynamic (PD) modeling and simulation (M&S) have been

TABLE 1 | Cancer cell lines/patient-derived tumor tissues applied to the CAM assay.

	Type of cancer	References
Solid tumor cancers	Recurrent respiratory papilloma	[94]
	Urothelial carcinoma	[95]
	Pancreatic carcinoma	[96]
	Ovarian carcinoma	[97]
	Melanoma	[98]
	Osteosarcoma	[99, 100, 101]
Blood-based cancers	Multiple myeloma	[102, 103]
	Burkitt lymphoma	[104]
	Acute myeloid leukemia	[105]
	Follicular lymphoma	[106]

well-established useful tools in pharmacological studies [107]. Although PK/PD modeling and simulations have many significant advantages [99, 108], the chicken embryo model provides an invaluable, unmatched tool for studies where in vivo biological complexity is essential to the research question. PK and PD simulations are computational models based on theoretical assumptions and biological parameters; the chicken embryo model, on the other hand, provides a more accurate in vivo environment that allows researchers to study a drug in a living, developing organism. Furthermore, chicken embryos develop rapidly, and their organ systems are functional from the start, making them ideal for studying how drugs affect specific biological processes in the developing organism. While PK/PD simulations rely heavily on mathematical equations and pre-existing knowledge, the chicken embryo model provides a more direct observation of a living organism that can reveal unexpected biological interactions that cannot be predicted by simulations. Conclusively, the chicken embryo model shares many similarities in biological pathways with humans, making it a good model for studying human diseases and drug responses.

Rodents have been the most used animal model for studying the pharmacokinetics and pharmacodynamics of various drugs and chemicals. Due to the importance of the 3R (Replacement, Reduction, and Refinement) concept in animal experiments, the search for alternative animal models has become very important in this area of research as well. The chicken egg represents a closed system, meaning there is no excretion of the drug, resulting in prolonged exposure after a single application [13] and reduced demand for multiple applications. In this sense, the well-known 21-day fetal development of the chicken embryo, its independence from the mother, and its noninvasive, easy, and quick injection methods make it a great substitute for the current animal models. Still, the embryo represents an independent compartment within the egg, making it feasible for both pharmacokinetic and pharmacodynamic studies. It is surprising that the chicken model is not yet commonly applied to nonclinical neuropharmacological studies, considering its long tradition of use in the field of neurodevelopment. We thus propose that early nonclinical trials of novel medications could effectively employ the chicken cerebellum as an important tool for the study of neurodevelopment.

In this context, the already established chicken embryo model was investigated to gain a better insight into the basic pharmacokinetic processes of morphine and methadone distribution to the brain and lung of the developing chicken embryo on 13 EID, following injection into the allantois (onto CAM) of the egg [102]. Morphine and methadone, as well as their metabolites, were detected in both the brain and lung, with significantly higher concentrations in the lung, suggesting the presence of a functional BBB in the developing chicken embryo from 13 EID. It was shown that brain distribution of morphine followed first-order absorption with transit compartments and linear elimination, with concentrations linearly dependent on dose. A pharmacodynamics endpoint was the downregulation of mu opioid receptors after methadone, but not morphine. In addition, fertilized chicken eggs has also been used as an alternative model to study the distribution of antiepileptic drugs into the developing chicken embryo brain at two developmental stages, 13 EID, when the BBB is less well developed [13], and 16 EID, when the CNS is more mature [7]. It was shown that valproic acid, as well as lamotrigine injected into the egg, rapidly distributes to the brain of the chicken embryo and reaches the CNS at human-relevant concentrations. In addition, lamotrigine concentrations were higher when injected at 13 EID than E16 EID. Similar injections led to a decrease in Pax6 level in the cerebellum [103]. It was confirmed that the chicken embryo model could be a suitable alternative animal model for preclinical studies of the distribution and effect of various drugs, characterized by high reproducibility and low time and financial costs. Knowledge of the pharmacokinetics of individual drugs and their behavior in the body is essential for the study of neurodevelopmental toxicity, especially about the concentrations that reach the brain at different developmental stages. One of the other disadvantages is the limited pharmacokinetics of some drugs. These are mainly insoluble compounds that cannot be administered in food or water. In the chicken embryo model, unlike the mouse model, it is not possible to administer drugs per orally, so all test substances must be dissolved before injection [12]. In addition to these limitations, there are other differences in drug metabolism and the immune system between chickens and mammals. This often means that the testing of various drugs has to be done in rodent models, and the chicken egg can be considered a suitable model that meets the 3R criteria before using the mouse model.

To study the distribution of persistent organic pollutants (POPs) to the fetal brain, chicken embryos were exposed to a POP mixture of 29 different compounds at 13 EID [109]. The concentrations of POPs in the brain were subsequently analyzed 0.5, 1, 2, 4, 6, 24, 48, and 72 h after administration. After administration of the mixture, up to 27 of the 29 compounds were detected in the developing brain during at least one of the time points analyzed. The concentration of the analyzed compounds reached a maximum value, followed by a decrease

over time. Similar studies showed dysregulations in the cerebellum [110]. These studies demonstrated that the chicken is a suitable model for investigating the distribution and effect of POPs in the developing brain at concentrations relevant to humans.

Another example of using a chicken embryo model to study the distribution of an environmentally toxic substance to specific organs is the study by Rutkiewicz and Basu [111]. This study evaluated the distribution of methylmercury in tissues during different developmental stages. The authors found that the distribution to the soft tissues of the embryo and hatchlings was similar to that of older birds, with higher total mercury concentrations in the liver and kidney than in the heart, muscle, and brain. The highest concentrations were detected in feathers and unabsorbed yolk. These findings suggest that embryos may accumulate certain concentrations of mercury in the kidneys and brain, which are known to cause the renal and neurotoxicity seen in older birds. However, the concealment of mercury in the liver and excretion into rapidly growing feathers may provide some protection.

There are also several studies that use chick embryo as a model for in ovo embryotoxicity on healthy embryos [112, 113] as well as drug tests on tumor-bearing chicken embryos like Uto et al. (2019), where they tested the tumor growth inhibitory effect of a radiosensitizer, etanidazole, by using chicken embryo [114]. In this study, transplanted mouse mammary carcinoma EMT6 cells and mouse colon cancer colon26 cells on chick embryos were evaluated for the pharmacokinetics of the drug. The chicken embryo is also a good model for studying cancer metastasis. For instance, Augustine and colleagues injected green fluorescent protein (GFP) expressing cancer cells and observed the migration of these cells to different organs [115]. All of these studies highlight the potential of the chicken embryo model in pharmacokinetic and pharmacodynamic studies, especially in development and disease.

In the last couple of years, some studies have used chicken embryos to make intestinal organoids, also called “mini guts” [116, 117]. Despite the high variation in organoid models in general, intestinal organoids derived from chick embryos give high reproducibility, suggesting they are a good model for gut studies, with the possibility of other organoid models that would be crucially useful in disease studies [118]. Taken together, all these studies confirm that the chicken egg is a valuable animal model for investigating the distribution and exposure of substances and drugs in various tissues of the developing fetus, and also lay the foundation for future drug safety and developmental toxicology research as well as pharmacology studies in the chicken egg model [118].

6 | Conclusion and Future Perspectives

This review summarizes the potential and limitations of the developing chicken as a promising alternative in vivo model in development and disease. The number of studies using chicken as a model system has been increasing, with more than 700 new research studies in the last 5 years. We present a number of studies describing the chicken embryo and its role in neurodevelopment and neurological disorders, in pharmacology (pharmacokinetic and pharmacodynamic studies), and as a cancer model, demonstrating that it is a 3R-friendly, easily accessible, simple to implement, cheap, reproducible, and above all, reliable model that can provide valuable biological answers.

Many experts from various scientific fields strongly support the use of the chicken model, applying the substance of interest onto different regions of CAM, due to the need to minimize the use of animal models, which often require a large number of animals in individual groups, which is often financially and time-consuming. For example, as mentioned above in Garcia et al., 2021, they focus on the immune system development and function within the chicken embryo, comparing it to humans. They offer an in-depth comparison of the chicken and human immune systems, detail immune organs and cell types as well as explain adaptive and innate immune processes, cytokine functions, and evolutionary differences. When conducting animal research, it is also essential to consider the ethical implications of using animals in experiments, as they are often subjected to various procedures that may involve pain, fear, or even harm. Therefore, researchers who use live animals in research must strive to minimize the impact of animal research by following the principles of the three R's: reduction, replacement, and refinement. Even though the genome size of avian is one third of humans, and only 60% of chicken genes have a traceable single human ortholog, avians have approximately 20 000–23 000 protein-coding genes, which is similar to mammals [119, 120]. Although, as mentioned above, the presented chicken model has some limitations that may be reflected in the unsatisfactory results of some clinical studies, it may represent a promising alternative to the often-used animal models. Despite the advantages of this model such as high availability, short incubation time, exact control of the number of embryos with defined developmental staging, and numerous improvements, one of its disadvantages is the lack of imaging and evaluation protocols to better document individual processes. Therefore, more studies are needed to make chicken a better preclinical test for the evaluation of biological processes, as well as the fact that substances and their metabolites cannot be eliminated from the egg during the experimental period, unlike other models. Despite a closed system, without any excretion of the substance [13] a classical pharmacokinetic profile was previously observed both for environmental pollutants [110] and for medications [7, 102] limiting the prolongation of the exposure and suggesting the use of the chicken embryo model also for pharmacokinetic studies. Because neither the drug nor its metabolites can escape from the egg, there must be an extra-embryonic compartment(s) serving as a sink in the egg. Based on the above, further studies are needed to make the chicken a better preclinical test for evaluating biological processes. Last but not least, it is possible to further investigate other scientific approaches on the chicken embryo model. In drug development, there is a huge demand for specific toxicity and in vivo efficacy and preclinical tests for the approval of new drugs, such as nanoformulations and nanocarriers for several drugs. The chicken embryo is certainly a promising model for investigating wound healing, fetal tendon healing, and microbiota-gut-brain axis among others. With the variety and flexibility of experimental methods available for the chicken embryo model, it is very clear that this innovative model system will continue to be at the center of developmental research for years to come.

Author Contributions

O.K.-A. and Z.D. carried out the experiment and collected the data. O.K.-A., Z.D., and R.E.P. wrote the manuscript.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Data will be available upon request from the authors.

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