Noninvasive Monitoring of Chick Development In Ovo Using a 7T MRI System From Day 12 of Incubation Through to Hatching

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Abstract

Purpose: To determine whether mild cooling of the egg reduces movement to the point where an ultra-high-field (7T) MRI system can be used to noninvasively monitor chick growth in ovo from 12 days incubation through to hatching.

Materials and Methods: Group A eggs were incubated at 37.5°C for 21 days. Group B eggs were removed from the incubator on days 12, 15, 17, 18, 19, and 20 of incubation, cooled for one hour, and then returned to the incubator. Group C eggs were cooled as for group B and then individually imaged for 25 minutes using a 7T MRI system before being returned to the incubator. The average size (volume) of the heart, liver, and brain at each stage of incubation was estimated from the T2-weighted images and compared with existing values in the literature.

Results: The combination of cooling and MRI significantly reduced chick movement to allow excellent image acquisition at each stage of incubation. Repeated cooling and/or MRI did not significantly slow down or arrest the development of the chicks in either of the experimental groups.

Conclusion: MRI provides a powerful noninvasive tool to study chick development and the growth of individual organs, including the brain, liver, and heart, in ovo from 12 days' incubation.

Key Words: 7T MRI; safety, chick embryo; fertile eggs, development in ovo

INTRODUCTION

THE AVIAN EMBRYO occupies a unique position among higher vertebrates in that it provides an excellent model of embryology. This is because all of the developing chick's requirements, with the exception of oxygen and heat, are provided by the egg contents and the surrounding eggshell. The first preserved account of a detailed description of the chick embryo is credited to Aristotle (384–322 BC), a Greek philosopher. Today developing eggs remain widely used by workers in both the pure and applied sciences.

There are several approaches to studying the dynamics of chick embryonic development and growth (1), but most require the embryos to be sacrificed at different stages of incubation to allow body size and individual organs to be measured. An alternative approach is to make repeated observations on the same embryo using noninvasive imaging techniques, such as magnetic resonance imaging (MRI). The advantage of this is that the number of embryos needed to attain statistical significance is significantly reduced, and repeated observations can be made on the same chick throughout the incubation process and subsequently related to the final phenotype at hatching (2). Another advantage of MRI over other noninvasive technologies, such as ultrasound, is that the presence of the eggshell does not present a problem in terms of acquiring images of the interior of the egg. Nevertheless, there are only a few accounts of MRI being used to image fertile eggs, and most of these relate to the initial stages of incubation (3– 6). Chick movement in the later stages of incubation has made imaging much more problematic, particularly beyond the 10th day of incubation (2,7).

In recent years MRI techniques have advanced rapidly, and as a result the signal-to-noise ratios (SNRs) have increased and scanning times have dramatically decreased. The challenge or aim of the current study was to test the feasibility of using a state-of-the-art ultra-high-field (7T) MRI system to monitor chick growth in ovo from the 12th day of incubation through to hatching. To minimize motion artifacts the eggs used in these experiments were cooled for one hour at 4°C in a refrigerator prior to imaging. Besides the problem of chick motion, the bioeffects of repeatedly exposing chick embryos to moderate short-term cooling and then high magnetic fields are also unknown. Another aim of this study was therefore to see if repeatedly cooling and/or imaging developing chick embryos was detrimental to their survival, growth, and hatching success.

MATERIALS AND METHODS

Animals and Treatments
Thirty broiler breeder eggs weighing 50–55 g were obtained from a commercial hatchery and placed in a digital tabletop incubator (Brinsea Products Ltd., Standford, UK). All eggs were laid on the same day and were stored for three days prior to the beginning of the experiment. After the eggs had been incubated at 37.5°C for six days they were "candled" (i.e., light was shone through them using a hand-held light source) to check if they were fertile and developing normally (as revealed by the shadows of the internal structures). Three eggs were removed from the incubator at

this time (one was cracked, one was infertile, and one showed signs of bacterial contamination). The remaining 27 eggs were then divided into three groups of nine eggs and treated as follows: Group A eggs were used as a control and left in the incubator for the full duration of the experiment (21 days). Group B eggs were removed from the incubator on days 12, 15, 17, 18, 19, and 20 of incubation, cooled for one hour in a refrigerator set at 4°C, and then returned to the incubator. Group C eggs were cooled as for group B on days 12, 15, 17, 18, 19, and 20, but were then individually imaged for 25 minutes before being returned to the incubator. Throughout the experiment, egg weight loss was monitored and the incubation temperature and relative humidity were adjusted to optimal conditions (in accordance with the incubator manufacturer's recommendations). Candling was repeated on days 12, 15, and 18 to check the viability of the embryos in both experimental and control groups. From day 20 the eggs remained undisturbed in the incubator so that the hatching success rate and average hatching time could be monitored.

Imaging

MRI was carried out using a 7T Bruker BioSpec 70/30 system (Bruker, Germany) together with a 120-mm inner- diameter actively shielded gradient set (400 mT/m maximum gradient) and a 72-mm birdcage volume resonator. The eggs were placed into a custom-built polystyrene holder that was suspended within the resonator/ magnet in order to minimize vibrations arising in the gradient coils. For the purpose of this investigation, when high-contrast/high-resolution images were a prerequisite, T2-weighted imaging was carried out using a multispin, multiecho pulse sequence (TR/TE = 6424/56 msec, in-plane resolution = 195 μm, slice thickness = 0.5 mm, interslice distance = 0.2 mm). Sixty slices were required to cover the entire egg, with a total acquisition time of 25 minutes per egg. T2- weighted images were chosen because they produced high signal contrast between the organs of interest, making them readily identifiable. T1-weighted images acquired in 3D mode provided higher-resolution images, particularly through plane, but produced a much reduced signal contrast, which made reliable organ delineation in the subsequent analysis very difficult.

Image Analysis

Different tissues and organ systems were identified on the basis of their anatomical position and signal contrast with other structures. For demonstration purposes, measurements of the volume of the heart, liver, and brain were subsequently carried out with the use of Bruker ParaVision software by the same observer, who used an electronic cursor to manually delineate each organ in each slice of the data set. The software then automatically integrated these areas over the slices to produce an estimate of the heart, liver, and brain volume for each egg ($N = 9$) at 12, 15, 17, 18, 19, and 20 days of incubation. These values were then averaged to give an indication of incubation time-dependent changes in the volume of each of these organs.

RESULTS
T2 Imaging of Chick Embryos
Examples from T2-weighted MRI multislice scans obtained at days 12, 15, 17, 18, 19, and 20 of incubation are shown in Fig. 1(a–f). Examples of the complete 60- slice

acquisitions for each of these time points can be viewed at
www.gla.ac.uk/7tmr/chickegg.htm.

At 12 days' incubation the chick is very small, and most of the available space is taken up by the yolk and the extraembryonic membranes and their associated fluid compartments [Fig. 1(a)]. The air sac is also very prominent. The most distinguishing features of the embryo are the eyes, brain, and heart, which serve as useful points of reference. By day 15 the chick has grown considerably and feathers can be visualized [Fig. 1(b)]. Many of the other organ systems are discernible, including the liver, gizzard, intestinal loops, and kidneys. The dorsal border of the lung tissue can also be seen embedded in the vertebral ribs. From the 17th day of incubation the chick's orientation changes in such a way that the head progressively moves down towards the air sac [Fig. 1(c–f)] The depressed state of the air sac in the images obtained from the later stages of incubation confirms that there must be considerable pressure exerted on this compartment as the chick continues to grow. The albumen mass is also no longer visible and the yolk sac surrounding the rapidly growing chick decreases in size. In the image corresponding to 20 days' incubation [Fig. 1(f)] the chick has just "pipped" (i.e., its head has penetrated through into the air sac at the blunt end of the shell). At this stage the chick actively begins to breathe, and the yolk sac becomes reabsorbed into the abdominal cavity via the naval in readiness for hatching.

MRI Estimates of Organ Sizes and Differential Growth Profiles
The MRI volume estimates ($N = 9$, mean ± SD) of the heart, liver, and brain at 12, 15, 17, 18, 19, and 20 days of incubation are shown in Fig. 2. The volume of the liver is proportionately greater than that of both the brain and heart at each time point. As these data come from the same nine individuals, it is possible to gain an appreciation of how these organs change in size over time. All three organ systems undergo the greatest increase in size between the 12th and 17th days of incubation. The low variance at each time point suggests that the MRI volume estimates are reproducible between individuals.

The second data set shown in Fig. 2 corresponds to published weights (in grams) of the same organs obtained by sacrificing individuals at each respective time point (8). These data serve as a useful benchmark with which to compare our MRI volume estimates. For the liver and brain there is a good correlation to the published data, but this is not the case for the heart.

MRI and Chick Survival
The survivability and hatching success of the chick embryos were not adversely affected by the cooling treatment (group B) or by the combination of cooling and imaging (group C). With just one exception (group B), all of the experimental eggs hatched within four to six hours of the controls (group A) on day 21. Hatchability Hatchability (number of hatched eggs/total number of eggs incubated) was calculated to be 86.7%, which is typical of the expected hatchability values for the Ross 308 broiler breeder eggs (www.aviagen.com) used in this study.

DISCUSSION

A pilot study carried out using the 7T MRI system prior to this experiment confirmed that if fertile eggs are not cooled prior to imaging, the image quality will be poor due to excessive chick movement from the 12th day of incubation. In the current study we found that cooling the eggs for one hour in a refrigerator set at 4°C prior to imaging significantly reduced chick movement, and as a result the MR image acquisitions were, with few exceptions, excellent. In addition, we were able to show that repeated moderate cooling alone or in combination with MRI did not significantly slow down or arrest the development of the chicks (groups B and C). With only one exception (in group B), all of our treated eggs hatched on day 21 within a few hours of one another and the untreated controls (group A). Moreover, there was no obvious pattern to the timing of hatching among the groups. We therefore feel confident that the repeated exposure of fertilized eggs to the electromagnetic fields of an ultra-high-field 7T MRI system did not adversely influence the survivability of the chick embryos used in our experiment. Though the number of eggs was relatively small, this study directly contributes to the general literature of negative results regarding the direct biological effects of MR on the tissues of the body, which were recently reviewed (9). The apparent lack of ill effects on the developing chick embryo may also be regarded as adding to the literature of negative results pertaining to the effects of MR on human pregnancy.

The change in the postural twist of the chick over time meant that it was not possible to image the chicks or their organs in the same orientation in a single scan on consecutive days. Nevertheless, the yolk, air cell, limbs, spinal column, and structures of the head, including the brain, eye, and beak, could all clearly be defined in the T2-weighted images associated with each 60-slice acquisition. The heart, liver, gizzard, intestine, lungs, kidneys, and pectoral muscles, plus many of the major blood vessels, were also easily identifiable [Fig. 1(a–f)].

For illustrative purposes the average volume or size ($N = 9$, mean ± SD) of the heart, liver, and brain at 12, 15, 17, 18, 19, and 20 days of incubation were estimated using the Bruker ParaVision software program (Fig. 2). According to these data the heart, liver, and brain grow most rapidly between days 12 and 17 of incubation. These findings are consistent with the changes in overall chick size, as illustrated in Fig. 1(a–f). As the MR images obtained in this study did not "cut" these organs in the same orientation each time, it could be argued that there will be errors in these measurements introduced by the so-called "partial-volume" effect (i.e., individual image pixels containing contributions from more than one tissue can reduce the clarity of the organ boundary). Errors introduced by the partial- volume effect can be reduced by acquiring images at a higher resolution, or by acquiring an image set oriented individually for each organ. However, the low variances observed in our measurements (Fig. 2) suggest that the partial-volume effect was of little consequence in our study. The MRI-estimated volumes for the brain and liver were also comparable with published weights for each stage of incubation (8). The former were obtained by continuously monitoring the same animals, as opposed to sacrificing multiple animals at each time point, and thus they probably give a more realistic impression of the differential growth of each of these organs over time. The MRI volume estimates for the heart were considerably different from those obtained by directly sacrificing chicks and weighing the heart; however, this is probably due to

the fact that blood within the heart chambers is inclusive in the MRI volume estimate of this organ. It might have been possible to overcome this discrepancy by using additional fast low-angle shot (FLASH) or fast imaging with steady precession (FISP)-based cardiac sequences that would have allowed the cardiac blood volume to be separately estimated. However, such techniques usually require gating with respect to the cardiac cycle using ECG signals, which are difficult to obtain from chicks in ovo. "Real-time" imaging using ultrafast gradients may circumvent the need for gating in the future, but the limited resolution afforded may in turn impact the accuracy of the blood-volume calculation.

As previously stated, MRI has significant advantages over other, less invasive methods of monitoring chick development, as the number of embryos needed to attain statistical significance is reduced. The fact, as shown here, that repeated observations can also be made on the same chick throughout the incubation process, and then related to the phenotypic characteristics of the emerging chick, is also highly beneficial compared to more invasive investigative methods.

In conclusion, the developing avian embryo continues to be routinely used as an experimental model in a wide variety of science-based disciplines concerned with such diverse fundamental questions as the effect of nutritional or endocrine deficiencies on embryonic development and growth, disease transmission, and the development of vaccines. This study shows that a 7T MRI system can be used as a powerful investigative tool to noninvasively study the development and growth of chicks and individual organ systems in ovo from 12 days' incubation. Moreover, the image quality is exceptional, allowing sequential quantifiable measurements to be made on the same chick throughout the incubation process. In order to image the chicks successfully, however, it is necessary to cool the eggs for one hour prior to imaging. The fact that 95% of the experimental eggs in groups B and C went on to hatch successfully suggests that treating the eggs in this way does not adversely affect the prenatal growth and development of the chick embryos. Additional studies are therefore currently being conducted to determine whether cooling is necessary before day 12 of incubation, and thus determine whether MRI can be used to continuously monitor chick development and growth from day 0 through to hatching. Studies of this type are pivotal to improving our understanding of early developmental processes and will help to answer key questions, such as how the extraembryonic membranes and egg contents become partitioned during the early stages of embryonic development in vivo (10). Further experiments are also being planned to ensure that postnatal chick growth is also unaffected by exposure of chick embryos to these treatments.

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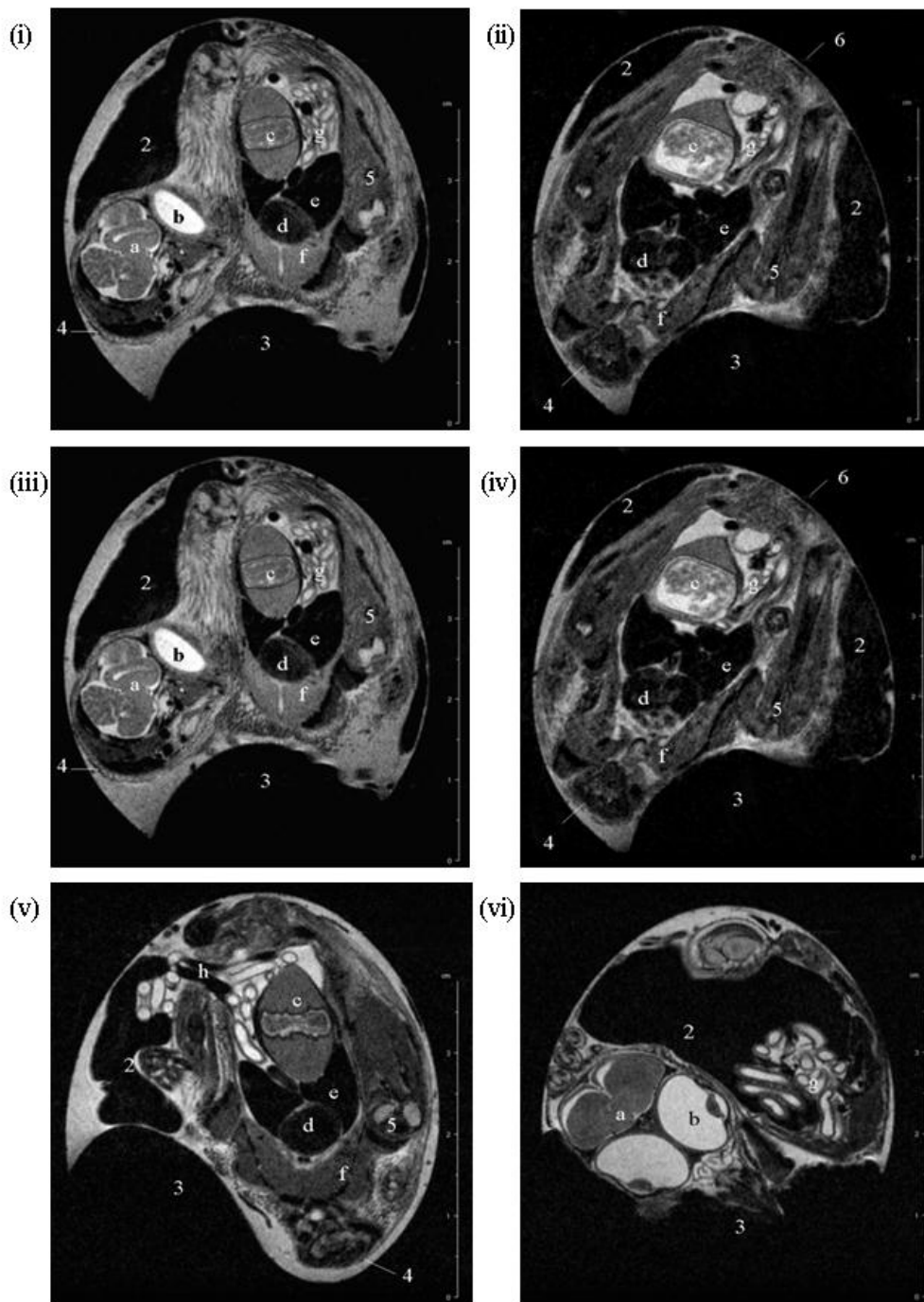
Figure 1. Representative examples of T2-weighted multislice scans of chick embryos in ovo at (**a**) 12, (**b**) 15, (**c**) 17, (**d**) 18, (**e**) 19, and (**f**) 20 days of incubation. 1 = albumen; 2 = yolk; 3 = air sac; 4 = head; 5 = limb; 6 = rump; a = brain; b = eye; c = gizzard; d = heart; e = liver; f = pectoral muscles; g = intestine; h = umbilical vessels. The complete scans can be viewed at www.gla.ac.uk/7tmr/chickegg.htm
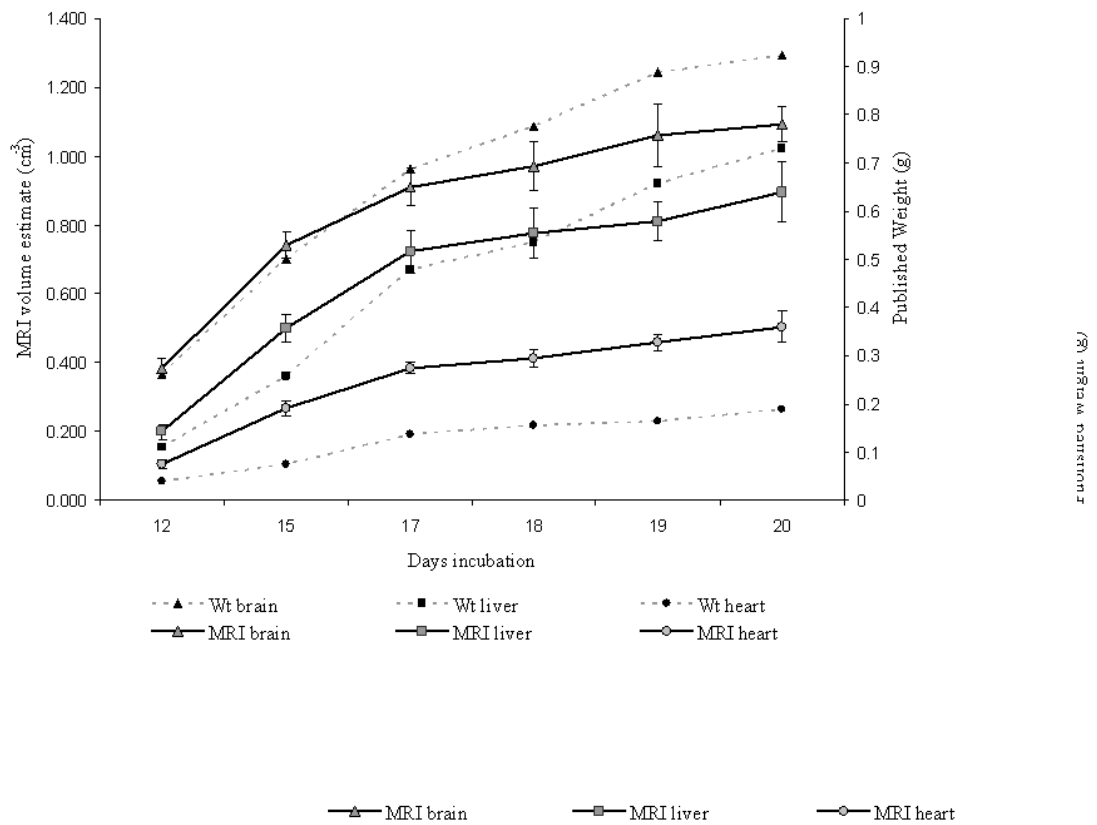
Figure 2.
MRI volume estimates of the heart, liver, and brain at days 12, 15, 17, 18, 19, and 20 of incubation (*N* = 9, mean ± SD). Published weights (in grams) for these organs derived by invasive techniques are also presented to allow comparisons to be made with the non-invasive estimates.