



## Short communication

# Ethical Euthanasia and Short-Term Anesthesia of the Chick Embryo

Ewa Aleksandrowicz and Ingrid Herr

Molecular OncoSurgery, Section Surgical Research, Department of General and Transplantation Surgery, University of Heidelberg and German Cancer Research Center (DKFZ), Heidelberg, Germany

### Summary

Fertilized chicken eggs are employed as an alternative to mammalian models. The chorioallantoic membrane (CAM) of the chick embryo is widely used for examination of angiogenesis, xenotransplants and for virus production. Unfortunately, it is mostly not taken into account that the chick embryo's ability to experience pain starts to develop at day 7 of incubation. In our view, this model is only in accordance with the 3R principles if an appropriate anesthesia of the chick embryo in potentially painful procedures is provided. Although many experimental approaches are performed on the non-innervated CAM, the euthanasia of the embryo strongly requires a more humane technique than the commonly used methods of freezing at  $-20^{\circ}\text{C}$ , decapitation or *in ovo* fixation with paraformaldehyde without prior anesthesia. However, protocols describing feasible and ethical methods for anesthesia and euthanasia of avian embryos are currently not available. Therefore, we established an easy and reliable method for the euthanasia and short-term anesthesia of the chick embryo.

Keywords: chick embryo anesthesia, chick embryo euthanasia, animal alternative model

The chick embryo and its chorioallantoic membrane (CAM) have been used in biomedical research for over 100 years (Ham-burger and Hamilton, 1951; Ribatti, 2004) and are frequently used as replacement of experiments performed on mammals. Study areas in which this model is employed include tumor biology (Ribatti, 2014), angiogenesis (Ribatti, 2008), pharmacology (Vargas et al., 2007), regenerative medicine (Coleman, 2008), teratology (Smith et al., 2012), infectiology (Jacobsen et al., 2011) and allergology (Slodownik et al., 2009). Experiments performed on avian embryos seem to be ethically more tolerable than those on rodents; this is reflected by the fact that unhatched birds are not considered living animals by national legislations worldwide. However, nociception in birds is similar to that in mammals (Lierz and Korbel, 2012) and there is consensus among scientists that avian embryos gain the ability to experience pain at a certain point of development. This capacity develops stepwise, beginning at day 7 of incubation (Rosenbruch, 1997). At day 13, the chick neural tube has developed into a functional brain and the animal is fully conscious a few days prior to hatching (ACUC California State Polytechnic University, 2012; IACUC University of Louisville, 2012).

In view of this, experiments on chick embryos can only be considered alternative methods to mammalian models when special

attention is paid to minimize pain and distress of the embryo. Investigations in which embryos older than day 7 and especially older than day 13 are used require the consideration of appropriate anesthesia. Moreover, even if experiments are only performed on extraembryonal structures (like the CAM), which are not innervated, the animal should be euthanized by a humane method at the end of the experiment. In regard to the American Veterinary Medical Association (AVMA) guidelines on euthanasia (AVMA, 2013), the applied technique should result in rapid loss of consciousness followed by cardiac or respiratory arrest and finally a loss of brain function. Pain and distress prior to the loss of consciousness should be minimized.

The commonly applied procedure to end experiments on fertilized eggs is freezing of the whole egg containing the embryo. This technique is not listed among the acceptable methods in the AVMA guidelines for embryos older than day 10 of incubation. Bird embryos that have attained  $> 50\%$  incubation should be euthanized by similar methods used in avian neonates, such as anesthetic overdose, decapitation or prolonged ( $> 20$  minutes) exposure to  $\text{CO}_2$  (AVMA, 2013). However, concerns are now being raised whether decapitation fulfills the criteria for a gentle and easy death, as conscious awareness may persist for up to 29 seconds in the disembodied heads (Bates, 2010). While  $\text{CO}_2$

Received October 3, 2014;  
Accepted January 14, 2015;  
Epub January 16, 2015;  
<http://dx.doi.org/10.14573/altex.1410031>



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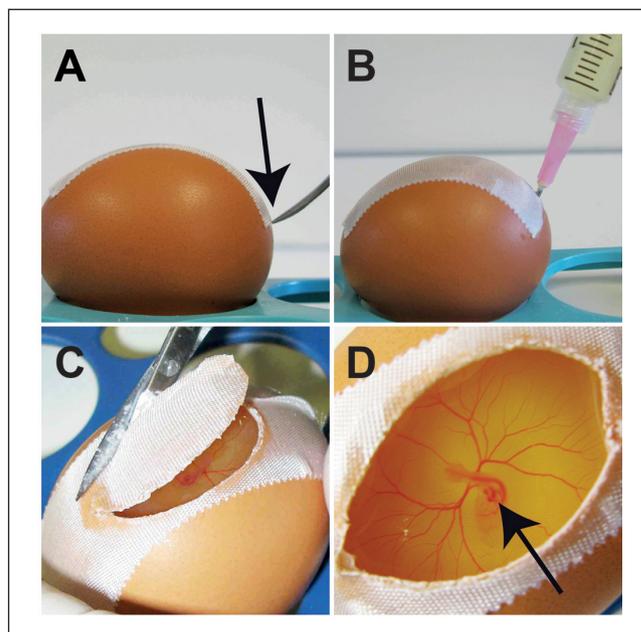


exposure has long been used as a method for euthanasia, questions have arisen that this practice may not be characterized as a humane method, as there is sufficient evidence that exposure to CO<sub>2</sub> is painful and may cause onset of asphyxia while the animal is still conscious (Conlee et al., 2005). Also, the required CO<sub>2</sub> dose may be difficult to adjust, as neonatal birds are more acclimated to high CO<sub>2</sub> concentrations (AVMA, 2013). In some publications the fixation of the CAM with paraformaldehyde without previous anesthesia or euthanasia of the embryo is reported. In this case, there is emerging evidence that the embryo dies a painful death.

According to the AVMA guidelines, animal welfare should be the main factor taken into consideration when choosing an appropriate method of euthanasia. In view of this, anesthetic overdose should be the method of choice. Unfortunately, protocols on how to anesthetize and/or euthanize avian embryos in a practicable and humane way are not available. Recently, the application of 2,2,2-tribromoethanol and urethane/ $\alpha$ -chloralose directly onto the CAM to prevent motion of the embryo was reported (Heidrich et al., 2011). However, anesthesia was not an objective of this study and there is no scientific evidence that the administration of these agents via this particular administration route results in a deep anesthetic state of the embryo. Heidrich et al. also used isoflurane, but the use of this agent requires expensive equipment, which may not be feasible for experimental approaches in which anesthesia is not the crucial objective. As we routinely use the chick CAM for cultivation of human cancer xenografts, we saw the need to develop a feasible and reliable method for the euthanasia of the embryos. According to the AVMA guidelines on euthanasia (AVMA, 2013), the intravenous injection of a barbituric acid derivative is the quickest and most reliable means of euthanizing birds. Thus, here we established a fast and easy method for the *in ovo* euthanasia of the chick embryo via administration of pentobarbital into the extraembryonic vascular system. The application of this method resulted in an immediate surgical anesthetic state in all examined embryos, which was maintained up to the time of death. Interestingly, 40% of the embryos, which at that time point were in the 15<sup>th</sup>, 17<sup>th</sup> or 18<sup>th</sup> day of embryonic development, were anesthetized but alive for longer than 5 min. Therefore, this technique can also be used for short-term anesthesia for experimental procedures in the living embryo.

Fertilized white Leghorn chicken eggs from a local ecological hatchery (Geflügelzucht Hockenberger, Eppingen, Germany) were stored for a maximum of 5 days at 10 ± 1°C. Prior to incubation eggs were washed with warm (40 ± 5°C) 70% ethanol. The eggs were incubated at 37.8 ± 0.2°C and a relative humidity of 50 ± 5% in digital motor breeders Type 168/D (Siepmann GmbH, Herdecke, Germany).

To gain access to the extraembryonic circulation, the eggs were opened on day 4 of embryonic development. At that time point the formation of the extraembryonic vascular network and the heart of the growing chick are already visible. On the 5<sup>th</sup> day of embryonic development, the CAM attaches to the inner eggshell membrane (Romanoff, 1960) and opening of the egg without rupturing this structure is no longer possible. The procedure was performed as described by Kunzi-Rapp et al. (2001) and Balke et al. (2011) with a few modifications. Briefly, the eggs were washed with warm (40 ± 5°C) 70% ethanol and placed on a six well plate



**Fig. 1: Opening of eggs on day four of embryonic development**

To allow intravenous drug administration, fertilized chicken eggs were opened at day four of incubation as described in the text. Representative photographs are shown (A-D).

in horizontal position. 2.5 x 5 cm Leukosilk<sup>®</sup> tape (BSN medical, Hamburg, Germany) was attached to the eggshell, covering the middle part and the rounded pole of the egg. A hole, circa 1 mm in diameter, was created at the round end of the egg above the air sac by knocking and gently drilling the eggshell with delicate curved scissors (Fig. 1A). Next, 3-4 ml albumen was aspirated with a 5 ml syringe and an 18G x 1 ½ needle. To avoid injury of the embryo, the needle was directed downwards (Fig. 1B). This step results in detaching and sinking of the embryonic and extraembryonic egg contents. Needles and syringes were changed regularly to avoid infections. Afterwards, a hole was created in the middle part of the egg, followed by enlarging the hole with scissors to a diameter of circa 1.5 cm x 2.5 cm in the taped area to avoid cracks in the eggshell (Fig. 1C). Viable embryos were identified by clear blood vessels and a beating heart (Fig. 1D). Finally, 1-2 ml of the previously removed albumen was re-injected into the egg and the window was sealed with tape.

Between developmental days 11 and 18 the embryos were euthanized via injection of Narcoren<sup>®</sup> (sodium pentobarbital 16 g/100 ml, Merial, Hallbergmoos, Germany) into the chorioallantoic vascular system. The anesthetic effect and the survival time following the drug administration were determined in 112 embryos. Prior to the injection of the narcotic, the window in the eggshell was widened until the point where the CAM meets the inner eggshell membrane. Next, blood vessels suitable for the administration of the barbiturate were identified on the CAM. Chorioallantoic blood vessels can be easily distinguished by color: light-red vessels are under low pressure and are suitable for drug injection. Dark-red vessels are under high pressure; these are not

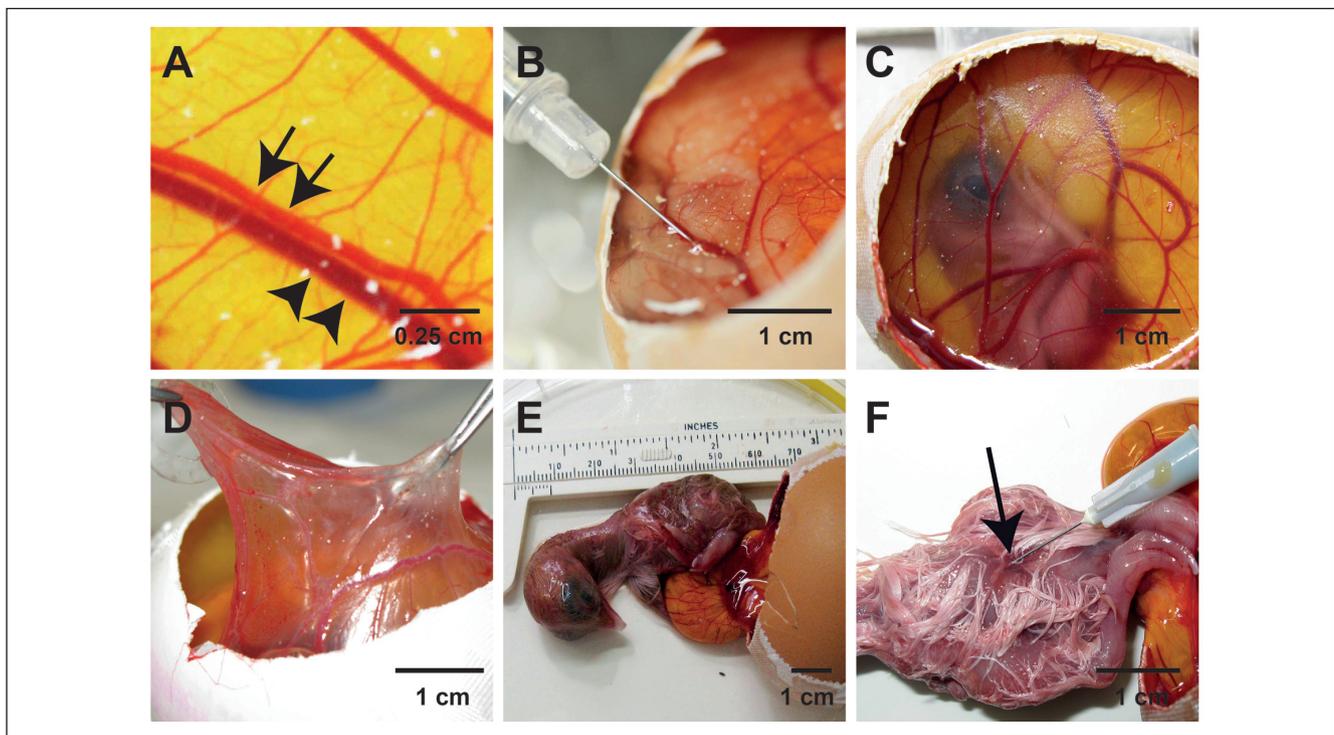
suitable for administration of substances as this would result in heavy bleeding (Fig. 2A). Thereafter, 0.02-0.05 ml Narcoren® was injected into a vessel of the CAM in each egg using a fine dosing 30G x 12 mm Omnican® insulin syringe with an integrated needle (B. Braun, Melsungen, Germany) (Fig. 2B). As dosage recommendations for chick embryos are not available, the dose is based on our experience. However, it is meant only as an orientation, as pentobarbital should not be given strictly in accordance with dosage recommendations but in accordance with the response of the animal (Löscher et al., 2014). Therefore, the embryo inside the egg should be observed carefully, while the intravenous drug administration is performed. After the narcotic was given, voluntary movements of the embryos as well as the pulsation of dark-red blood vessels were monitored inside the egg (Fig. 2C).

All embryos stopped moving inside the egg immediately after the injection of pentobarbital. In almost one third of the eggs the injection of the drug resulted in an immediate interruption of the pulsation of the extraembryonic blood vessels. Consequently, cardiac arrest in those embryos occurred immediately after this procedure. The embryos were observed in the egg for 1 min. Thereafter the CAM was lifted quite high to ensure that no parts of the embryo had been caught (Fig. 2D) and ruptured using delicate forceps. Finally, the embryo was gently taken out of the

egg (while care was taken not to rupture the navel) and placed in lateral position (Fig. 2E). Because the bird might still be fully conscious it should not be positioned in dorsal recumbency, as this impedes respiratory activity (Lierz and Korbel, 2012).

Occasionally, intravenous injection is not feasible due to the lack of accessible vessels on the CAM. In this case the bird was taken out of the egg and immediately euthanized via intracoelomic injection of 0.05 ml Narcoren®. To avoid injections in the air sac and ensure fast absorption of the drug, the intracoelomic injection was performed in the abdominal region of the bird (Fig. 2F). The depth of anesthesia, as well as the vitality of the embryos were monitored every 5 min until death. The cardiac activity was monitored via palpation of the bird's chest. The presence or the absence of corneal, palpebral and pedal reflexes, voluntary movements and responses to postural changes served as assessment criteria for the level of anesthesia. The examination revealed good muscle relaxation, as well as absence of the above-mentioned reflexes in all embryos.

Among the embryos, which were in the 15<sup>th</sup>, 17<sup>th</sup> or 18<sup>th</sup> day of incubation, 40% survived longer than 5 min, whereas 10 min after drug administration cardiac arrest could be determined in 93% of the embryos. Seven percent of the embryos survived for 15 min or longer (Fig. 3A). Among the 11-14 day old embryos

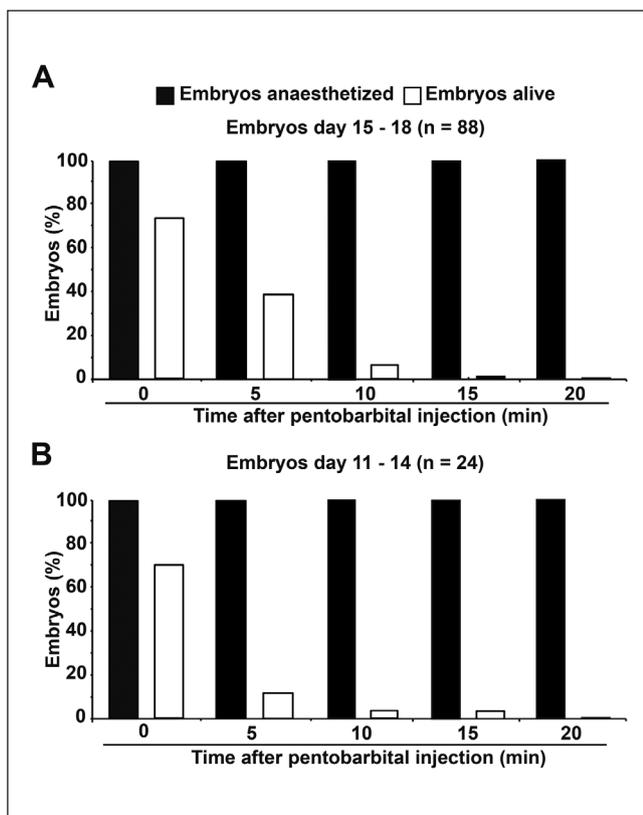


**Fig. 2: Administration of pentobarbital into the extraembryonic vascular system**

Narcoren® was administered intravenously into the chorioallantoic vascular network of 112 eggs containing embryos at day 11-18 of embryonic development. Representative photographs are shown. (A) CAM on day 18 of embryonic development. Light-red blood vessels (arrows) carry oxygen-rich blood, are under low pressure and are suitable for the injection of liquids. Dark-red blood vessels are under high pressure and are not suitable for drug administration, as this would result in heavy bleeding (arrowheads). (B) Pentobarbital is administered intravenously in the vascular system of the CAM using a fine dosing insulin syringe with an integrated needle. (C) Embryo is monitored inside the egg. (D) The CAM is lifted with delicate forceps and ruptured. (E) The embryo is taken out of the egg and placed in lateral recumbency. (F) If the intravenous injection is not possible due to the lack of accessible vessels on the CAM, the bird is euthanized via intracoelomic injection of pentobarbital. The correct position of the needle is demonstrated (arrow).

**Tab. 1: Minutes of survival of anesthetized chick embryos after intravenous injection of pentobarbital**

| Incubation day of egg | Total no. of embryos | No. of embryos with cardiac arrest at different time points (min) after pentobarbital administration |    |    |    |    |
|-----------------------|----------------------|--|----|----|----|----|
|                       |                      | 0  | 5  | 10 | 15 | 20 |
| 18                    | 77                   | 15   | 42 | 71 | 76 | 77 |
| 17                    | 1                    | 0  | 1  | 1  | 1  | 1  |
| 15                    | 10                   | 8  | 10 | 10 | 10 | 10 |
| 14                    | 9                    | 4  | 6  | 8  | 8  | 9  |
| 13                    | 9                    | 3  | 9  | 9  | 9  | 9  |
| 12                    | 3                    | 0  | 3  | 3  | 3  | 3  |
| 11                    | 3                    | 0  | 3  | 3  | 3  | 3  |

**Fig. 3: Time schedule of anesthesia and death of the chick embryo after pentobarbital**

Narcoren® was injected into the vascular system of the CAM in 112 eggs hosting chick embryos in the 15<sup>th</sup>, 17<sup>th</sup> or 18<sup>th</sup> (A) and the 11<sup>th</sup>, 12<sup>th</sup>, 13<sup>th</sup> or 14<sup>th</sup> (B) day of embryonic development. Subsequently after the administration of the drug the depth of anesthesia, as well as the vitality of the embryos were monitored every 5 min until the time of death. The embryos were considered as being anesthetized in the case of the absence of the corneal, palpebral and pedal reflexes, voluntary movements and responses to postural changes. The bird's cardiac activity was determined via palpation of the chest. The diagrams visualize the percentage of anesthetized and living embryos at different time points after the drug administration.

the survival time was shorter. Cardiac arrest was detected in 88% of all embryos 5 min after the barbiturate was given (Fig. 3B). However, one 14-day-old embryo survived for up to 20 min (Tab. 1). As mentioned above, in rare cases, the intravenous injection was not feasible and the birds were euthanized via intracoelomic injection only. Prior to the drug administration these embryos showed active voluntary movements, particularly of the beak and caudal extremities. This could be observed for up to 5 min after the intracoelomic injection of pentobarbital. Consequently, the intravenous route is the preferred option and should be performed whenever possible.

The intravenous administration of pentobarbital resulted in a prompt interruption of motility, loss of muscle tension and reflexes in all embryos. According to Lierz's and Korbelt's determination of anesthetic stages in birds (Lierz and Korbelt, 2012), the absence of the palpebral, pedal and corneal reflexes in combination with good muscular relaxation are evidence for a deep surgical anesthetic state.

Pentobarbital leads to a rapid loss of consciousness in warm-blooded animals (Löscher and Rogawski, 2012), is readily available in approved solutions (e.g., Narcoren®) and is less expensive than many other euthanasia agents. A disadvantage may be the bureaucratic burden, as the use of barbiturates is strictly regulated by national legislation in most countries.

If pentobarbital is not available, the use of other injectable anesthetic agents may be considered, such as a combination of ketamine and an alpha-2 agonist, i.e., medetomidine or xylazine. It must be noted that none of these is to be used as a monoanesthetic agent due to the insufficient analgesic potency in birds (Lierz and Korbelt, 2012). Based on our experience, in an average 18 day 10 g chick embryo the combination of 2 mg ketamine and 0.2 mg xylazine applied into the breast muscle leads to a good anesthesia and a rapid death within a few minutes. For longer survival times and/or intravenous administration routes the dose should be carefully adjusted based on the effect on the animal. While dosage recommendations for unhatched birds do not exist, it is always to be taken into account that young and small animals need relatively higher doses compared to adults. However, these alternatives to pentobarbital are not authorized for euthanasia, are more expensive, and the necessity of mixing two substances prior to the application is less practical.

In conclusion, there is no doubt that our method implies some administrative and time-consuming burdens, and to skip the anesthesia is the less complicated way. Still, to address experiments with avian embryos as alternatives to animal models in accordance with Russell's and Burch's principles of humane experimental technique (Russell, 1995), the minimization of animal pain and distress should be of higher value than the minimization of human effort. The intravenous administration of anesthetics into the CAM is a feasible method for euthanasia and short-term anesthesia of the avian embryo, which still maintains the balance between the ideals in medical ethics and the everyday reality in the laboratories.

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## Conflict of interest

None of the authors has a conflict of interest to disclose regarding the publication of the present manuscript.

## Acknowledgements

Our study was supported by grants from the Federal Ministry of Education and Research (BMBF 031A213), Heidelberger Stiftung Chirurgie, Stiftung für Krebs und Scharlachforschung, Hanns A. Pielenz Stiftung and Dietmar Hopp-Stiftung.

## Correspondence to

Ingrid Herr, PhD  
Section Surgical Research  
Im Neuenheimer Feld 365  
69120 Heidelberg, Germany  
Phone: +49 6221 56 6401  
Fax: +49 6221 56 6402  
e-mail: i.herr@dkfz.de