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In ovo therapeutics to improve gut health and efficiency in the broiler chicken

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In ovo *therapeutics to improve gut health and efficiency in the broiler chicken*
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INTRODUCTION

With almost 40% of the broiler chicken's life now being spent in the egg, this period of embryonic development is becoming increasingly important. Indeed this phase now represents a new frontier for the broiler industry, given that most of the efforts to date have concentrated on post hatch genetics, nutrition and management. There are, however, very few reports of attempts to influence the environment in the egg apart from very late (day 18) simple nutritional supplementation (Foye et al, 2006; Kornasio, 2011). We know that the environment in which embryos and fetuses develop, can have dramatic effects on their lifelong health and performance. This phenomenon is known as fetal programming. The aim of this project was to identify and develop targeted *in ovo* therapeutics that could be administered at key time points in embryonic development to enhance gut development and improve post-hatch performance and health.

APPROACH

DEVELOPMENTAL SERIES

To target particular windows of gut development we required a thorough knowledge of the timing of events in the development of the small intestine of the normal broiler bird. After searching the literature we were unable to find a full developmental histological series of the gut hence our first step was to develop one of our own (see CRC 2.1.2 Milestone Rpt 1). Since this report we have also managed to develop a full histological series of the small intestinal development of commercial layer birds to allow a comparison to be made between layers and broilers. We are currently preparing this information for publication.

OUTCOME: Full developmental, histological series of the small intestine of both broilers and layers made and analysed. Currently being prepared for publication as a comparative study.

***IN VITRO* ASSAYS**

The approach we took initially to test our selected candidate compounds was to develop and optimise an *in vitro* system whereby we could test potential nutraceuticals in a rapid and highly controlled manner. We established an *in vitro* cell proliferation organ culture assay using freshly dissected jejunal tissue from broiler chickens at embryonic day 15. At embryonic day 15 the jejunal villi are just forming and goblet cells are first apparent. The tissue was then exposed to the compounds for several hours while the cells were still behaving normally (DNA synthesis linear) which allowed us to quantify cell proliferation rates using the rate of BrDU incorporation.

Candidates tested included:

- Compounds from the pentose phosphate pathway (ribose, ribose-5-phosphate)
- Mitogenic agonists and antagonists (Lithium Chloride, Wnt agonist)
- TGF-β superfamily members (activin, follistatin)

- Cell proliferation substrates (glucose, L-lysine, L-threonine, taurine)
- Polyamines & their precursors – spermine, putrescine, arginine
- Folic acid & methionine metabolism intermediates (choline, betaine, L-methionine, S-adenosyl-methionine)
- Antiproliferative compounds (PDGF Tyrosine Kinase Inhibitor IV, Stem cell proliferation inhibitor, kaempferol

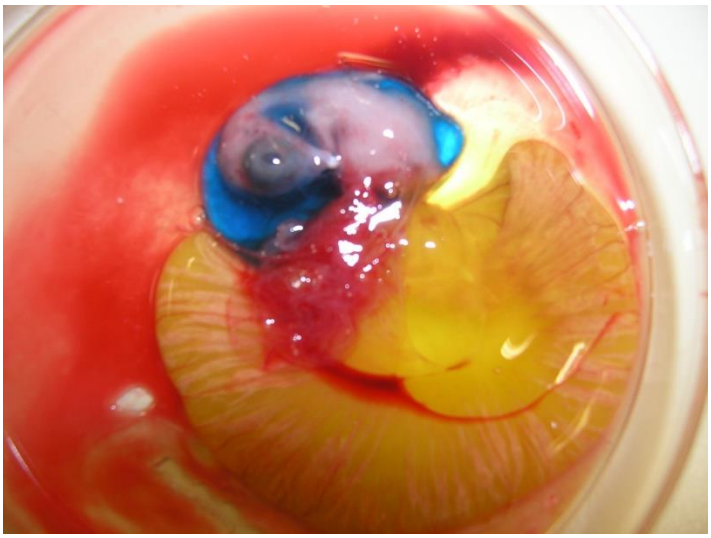
There were a number of compounds that significantly influenced cell proliferation and were considered of interest for further testing *in ovo*. However the nature of the *in vitro* assay meant that any compounds that might affect maturation of the gut (differentiation of cells) or require metabolism through the liver would not be identified by the organ culture system hence the next logical step was to start *in ovo* injection experiments.

***OUTCOME:* In vitro assay developed and optimized. Twenty (20) candidates tested for effects on jejunal cell proliferation, with several candidates having significant effects.**

EMBRYONIC DAY 9 *IN OVO* INJECTION MODEL DEVELOPMENT

We successfully developed and optimized a system for delivering compounds to the developing chicken embryo by injection into the incubating egg via the amnion, yolk sac or chorioallantoic membrance (CAM) at embryonic day 9. Eggs injected with control vehicle had hatchability rates similar to that of non-injected eggs (approx 90%).

A pilot test was completed using cortisol and metyrapone injected into the yolk sac or onto the CAM of eggs at embryonic day 9 to act as a positive control for our assay system. Literature has shown that cortisol injected into eggs inhibits embryonic growth (Karnofsky et al. 1951). We were able to replicate this result showing that our system was suitably responsive.



OUTCOME: A responsive in ovo injection method developed and optimized for delivery of candidates at embryonic day 9 to either the CAM, yolk sac or aminon of the fertilized egg with no detrimental effects on hatchability.

IN OVO TESTING OF CANDIDATES

Our *in ovo* system was used to test the following compounds injected **into the amnion** of fertile eggs at embryonic day 9: Lithium chloride, arginine and butyrate.

Arginine treatment at embryonic day 9 (E9) significantly increased ileal weight (34%) and length (23%) when measured at embryonic day 14 and also at day of hatch. These findings were supported with changes also found in the histology of the ileum. There was also a significant increase in the jejunal villus number in the chickens treated with arginine at E9.

Having shown that we could positively alter the development of the chicken gut by *in ovo* injection of arginine it was decided, in consultation with the CRC, that it would be beneficial to target treatments to an earlier embryonic time point so that the duodenum and jejunum would be less mature at the time of treatment and more likely to respond to treatment along with the ileum. (During chick development there is a distinct developmental wave from the cranial portion of the small intestine toward the caudal end).

OUTCOME: Three compounds tested using the in ovo E9 injection method. Arginine successfully increased jejunal villus number and the weight and length of the ileum when measured at E14 or day of hatch.

MODIFICATION OF *IN OVO* METHOD TO TARGET EARLIER DEVELOPMENT

Experiments were conducted to investigate various routes of administration (amnion, yolk and albumen) to target earlier chick developmental stages. Dose rates, diluent composition and various volumes of injectate were tested to find conditions to suit the early embryo.

In our hands it was not possible to inject the amnion before E9 without the accuracy of injection falling below acceptable limits. It is possible to inject the yolk after day 5 of incubation when the yolk starts to liquefy and has lost its central latebral core. Before day 5 the injectate enters the latebral core and travels straight to the nucleus of pander beneath the blastoderm and is often fatal.

After much experimentation it was found that we could inject arginine, dissolved in distilled water, into the albumen of pre-set eggs (day 0) with a volume of 100µl without affecting viability.

OUTCOME: It is possible to inject arginine into the albumen of fertilized eggs before setting without detrimental effects on chick viability.

IN OVO TESTING OF ARGININE INJECTED AT DAY 0

Arginine was injected **into the albumen** of pre-set eggs. This resulted in a significant improvement in hatchability (Controls 84% vs Arginine 100%). Arginine treatment also increased the weight of the liver, gizzard and bursa of fabricius at day of hatch. If the increase in liver weight is due to increased glycogen stores this would be beneficial especially for the time that chicks are held and transported without feed. It is reported that during this period of fasting the chick weight decreases rapidly and also has lasting effects on the bird's lifetime performance (Pinchasov and Noy, 1993; Noy and Sklan, 1999; Kornasio et al., 2011). It has also been shown that an increased gizzard weight correlates to a more efficient chicken (de Verdal, 2010).

OUTCOME: Arginine injected into the albumen at day 0 significantly increased the weight of the liver, gizzard and bursa of fabricius of the chicks. Given this result it would be logical to test the FCR of the treated chickens, if time permitted, in future experiments and to investigate chick reserves after a period of post-hatch fasting.

IN OVO TESTING OF POLYAMINES (INC. PUTRESCINE) INJECTED AT DAY 7

Pilot trials were done to determine if the polyamines (spermine, spermidine, putrescine and S-adenosyl-L-methionine) injected **into the air cell** at day 7 would alter viability, bodyweight and length of embryonic broiler chickens.

Day 7 was chosen to precede the known natural polyamine peak at day 12-17 that corresponds with organ growth and maturation (Lowkvist et al., 1985).

The polyamine putrescine proved to be the most promising compound so a full trial was done. Injecting **6µmoles of putrescine into the air cell of day 7 eggs** resulted in significant gross and histological changes in chickens at day of hatch and during the post-hatch period with no detrimental effects on viability or hatchability. These changes included:

- Increased weight and length of the gastrointestinal tract at day of hatch
- Increased weight of the gizzard at day of hatch

- Increased crypt depth of the duodenum at day of hatch
- Increased villus width and total villus area of the ileum at day of hatch (in fact there was an increase in villus height along the entire gastrointestinal tract although this did not quite reach statistical significance)
- Increased growth rate and bodyweight of female chickens up to 28 days of age*

  (* this experiment was confounded by unvaccinated chickens being exposed to IB via vaccinated birds being brought onto the same facility. There was evidence of a subclinical infection although growth rates etc. were above Cobb 500 standards. Perhaps this infection was enough to prevent optimal performance after 4 weeks of age)

OUTCOME: Despite the confounding factor (IB exposure) in this experiment the birds treated with putrescine at embryonic day 7 showed significant positive gross and histological changes to their gut and an increase in growth rate.

PUTRESCINE FCR TRIAL

Feed conversion ratio was assessed by performing an apparent metabolisable energy trial (AME) on chickens treated *in ovo,* **via the air cell** route, with putrescine at embryonic day 7. Digestion ability was also assessed by brush border membrane analysis (currently being run – results due 31/5/14).

The FCR trial showed:

- Significant improvement in hatchability of putrescine treated eggs
- Significant improvement in weight gain of male chickens over the 6 week grow out period
- Many positive trends that indicate improved digestion efficiency of treated male birds such as
  - 1.6% reduction in FCR
  - Increase in gizzard weight at D42
  - Overall reduction in total gut weight and length at D42 indicating improved efficiency (de Verdal et al., 2010)
  - Increased daily input (measured during the AME trial, D16-23) of on average 6.1g, yet daily output was on average only 1g more. This shows potential for treated birds to reach their target weight earlier.

OUTCOME: Putrescine injected into the air cell of fertile eggs at embryonic day 7 significantly increased weight gain of male chickens over the grow-out period. This appears to be due to a number of small improvements in gut morphology and digestive efficiency which act together to result in the increased weight gain.

POTENTIAL FOR DEVELOPMENT OF A COMMERCIAL APPLICATION OF THE COMPOUNDS FOR *IN OVO* INJECTION

The positive effects on growth and gut development from *in ovo* treatment with putrescine and arginine provide us with the evidence that both of these compounds have the potential for further development and commercial application.

Although we think *in ovo* application using the currently available 'Embrex Inovoject' technology would make it possible to administer either of these compounds at the desired timepoint (day 7 or day 0). An alternative and more practical approach would be to feed the target compounds to the broiler breeder hen. We also think that this approach should be used to test betaine, based on the findings of David Cadogan (Feedworks/Danisco) who demonstrated had a positive effect of betaine in the hen diet on hatchability. This approach has been the subject of a recent FRP put to the CRC for consideration.

Our research group has established a close relationship with HiChick, Bethel, SA, which would enable us to conduct a commercially- relevant trial manipulating levels of arginine, putrescine or betaine in the broiler breeder hen's diet with an opportunity to study the progeny of the manipulations. If the dietary addition of these compounds to the broiler breeder hen diet caused even a small gain in broiler productivity, similar to the changes we have noted in our *in ovo* administration, it would be readily adopted by the industry where a 2 point improvement in efficiency is worth approx. $13.5 million annually.

PUTRESCINE

Putrescine is a polyamine that has an essential role in cellular differentiation and proliferation. An increase in polyamine synthesis is followed by an increase in the rate of synthesis of DNA, RNA and protein (Heby, 1981).

Putrescine has been studied as a dietary additive for broilers, layers, ducks and turkeys. Very low levels included in the diet of broiler chickens have been shown to improve growth (Smith, 1990; Sousadias and Smith, 1995). In turkeys, when fed putrescine only during the starter period the growth rate in the grower phase was increased (Smith et al., 2000). In addition Girdhar et al., 2006 found turkey poults supplemented with putrescine had improved mucosal development of the small intestine and improved recovery from subclinical coccidiosis. Conversely putrescine fed to ducks was found to have a negative effect due perhaps to a greater sensitivity (Peng et al., 2010).

COST/AVAILABILITY

In ovo application

Putrescine is an attractive compound to adapt to a commercial application due to its availability and cost. Putrescine is readily available in Australia from chemical

companies such as Sigma. The current price for 100g of putrescine (98% pure) is $327. We treated each egg with 0.23µg. This equates to $0.000000765 per egg.

Our treatment involved injecting eggs in the air cell on day 7 (we believe this is the ideal time based on known natural spikes in polyamine levels in the developing embryo (Lowkvist et al., 1985)). In a commercial setting this would add an extra step to the incubation process. Current technology used for *in ovo* vaccinations, 'Embrex Inovoject' would be capable of injecting the solution. We have found throughout our experiments that removal of eggs for up to 20 minutes from the incubator has no detrimental effect on viability and hatchability.

However, we believe a more commercial approach would be to feed the broiler breeder hen. Although there have been no studies of putrescine feeding to layers or broiler breeders and the impact of this on levels measured in the egg, there have been studies of the effects of dietary putrescine on egg quality. Smith et al. (2000) found that feeding 0.1 or 0.15% putrescine to layers for 4 weeks resulted in a significant reduction in egg shell deformity. This indicates that dietary putrescine in the breeder hens diet has the potential to reach the egg.

Feeding of the breeder hen

There are a number of examples in the literature where compounds have been added to the broiler breeder hen diet with the aim of influencing progeny performance, results have shown varying success. Examples include L-carnitine (Kidd et al., 2005), Selenomethionine (Wang et al., 2011), yeast products (Kidd et al., 2013) and Betaine (Cadogan, unpubl data).

Due to the positive outcomes we have shown with *in ovo* putrescine treated eggs such as improved gut development and growth we think elevated putrescine levels in the egg achieved by feeding the broiler breeder hen targeted levels of putrescine will induce similar epigenetic effects.

Ideally a proof of concept trial would be conducted initially where breeder hens or even layers are fed putrescine in their diet at the concentration proposed and levels in the egg measured.

ARGININE

Arginine is another attractive epigenetic candidate. An essential amino acid, it is a known stimulator of growth and development in fetal and early life models in other species. Arginine is a precursor for the synthesis of polyamines, growth hormone, insulin like growth factor and nitric oxide (Chevalley et al., 1998; Torricelli et al., 2002). In neonates arginine is synthesized by gut epithelial cells, studies with neonatal piglets have shown that arginine supplementation improves intestinal integrity due to nitric oxide mediated vasodilatation and blood flow (Puiman et al., 2011).

COST/AVAILABILITY

In ovo application

Arginine has previously been investigated as an *in ovo* therapeutic in avian species, but only at later stages in incubation as an energy source during late development. In *ovo* administration of arginine in the amnion of duck eggs at 23d of age improved glycogen reserves (Tangara et al., 2010). In *ovo* administration of arginine in turkey eggs at 21 and 23d of age enhanced jejunal nutrient uptake and digestion (Foye et al., 2007).

A study using quail eggs showed promising results with the *in ovo* application of arginine in the air sac at day 0 resulting in significant positive changes in production and performance (Al-Daraji et al., 2011).

The current list price of 98% purity arginine from Sigma is $326 for 1kg. We used 15mg per egg thus the cost per egg if treated *in ovo* would be $0.00489.

Feeding of the breeder hen

We selected arginine for its potential to alter the growth and development of the intestine. This could possibly be achieved through its effect on GH and IGF-1, strong mediators of cell proliferation. Oral arginine administration has been reported to significantly increase serum levels of these factors in humans (Isidori et al., 1981; Hurson et al., 1995).

Silva et al., 2012 conducted a study investigating the effect of arginine supplementation on broiler breeder egg production and quality. They concluded that the highest egg production was obtained when the diet was supplemented with 1.262% arginine, with no effect on hatchability. This study did not follow the performance of progeny.

As with putrescine ideally a proof of concept trial would be conducted initially where breeder hens or even layers are fed putrescine in their diet at the concentration proposed and levels in the egg measured.

Following the proof of concept trails experiments would be conducted to test the feeding of these compounds to broiler breeder hens with progeny measurements taken. For a draft design of these experiments please see Milestone 6.

CONCLUSION

Both putrescine and arginine would be viable, potential *in ovo* therapeutic compounds with beneficial effects on broiler development and growth. Using a modified version of the current Embrex Inovoject system both compounds should be possible to inject in a commercial setting. Arginine could be injected into the albumen of the fertilised eggs pre-set (day 0). Putrescine could be injected into the air cell of fertilized eggs on day 7 of incubation. Even at current list prices of both compounds the amount required to be injected into each egg is not cost prohibitive. The injection of arginine or putrescine caused no detrimental effect on hatchability or chick viability. In fact putrescine significantly improved hatchability.
Given all this, *in ovo* injection using putrescine or arginine is a real possibility, however as outlined above we feel that an even better solution may be to feed the compound to the broiler breeder hens for transfer into the egg. This idea requires testing and has gone to the CRC as a full research proposal for consideration.

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