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1 **TITLE:**
2 Intranasal Immunization and Milk Collection in Studies of Maternal Immunization in New Zealand
3 White Rabbits (*Oryctolagus cuniculus*)
4
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17 **KEYWORDS:**
18 Maternal immunization, Intranasal immunization, Mucosal immunization, Milk collection, Rabbit
19 milk, *Oryctolagus cuniculus*.
20
21 **SUMMARY:**
22 This article describes and demonstrates the administration of intranasal vaccines and the
23 collection of milk from lactating rabbits (*Oryctolagus cuniculus*) as a means to assess mucosal
24 immunity in a translationally appropriate model of maternal immunization.
25
26 **ABSTRACT:**
27 Due to similarities in placentation and antibody transfer with humans, rabbits are an excellent
28 model of maternal immunization. Additional advantages of this research model are the ease of
29 breeding and sample collection, relatively short gestation period, and large litter sizes. Commonly
30 assessed routes of immunization include subcutaneous, intramuscular, intranasal, and
31 intradermal. Nonterminal sample collection for the chronological detection of the immunologic
32 responses to these immunizations include the collection of blood, from both dams and kits, and
33 milk from the lactating does. In this article, we will demonstrate techniques our lab has utilized
34 in studies of maternal immunization in New Zealand White rabbits (*Oryctolagus cuniculus*),
35 including intranasal immunization and milk collection.
36
37 **INTRODUCTION:**
38 Studies of maternal immunization and antibody transfer are invaluable for numerous reasons, as
39 this is the initial route of immunity transfer and subsequent protection from pathogens and
40 diseases in newborns and infants. Maternal immunization has the potential to positively impact
41 both maternal and infant/child health at the global level by reducing morbidity and mortality
42 associated with certain pathogens during this vulnerable period[1]. The main goal of this strategy
43 is to increase the levels of specific maternal antibodies throughout pregnancy. These antibodies
44 can then be transferred to the newborn and infant at levels sufficient enough to protect against

45 infections until their immune system is mature enough to adequately respond to challenges[1-3].
46 Previous work has demonstrated that higher antibody titers at birth are associated with either
47 complete protection or a delayed onset and reduced severity of numerous different infectious
48 diseases in the newborn, including tetanus, pertussis, respiratory syncytial virus (RSV), influenza,
49 and group B streptococcal infections[1-3].
50
51 In humans, maternal antibodies are transferred passively across the placenta and are also
52 transferred through the breast milk via nursing. Previous work has demonstrated that HIV-
53 specific IgA levels in human breast milk from mothers infected with the virus were associated
54 with reduced postnatal transmission of the virus, suggesting a protective role for breast milk anti-
55 HIV IgA[4]. Studies in nonhuman primates have demonstrated that immunization against HIV can
56 induce a significant antibody response in the breast milk, and although similar serum IgG
57 responses were induced following systemic versus mucosal immunization, mucosal
58 immunization induced a significantly higher IgA response within the milk[5,6].
59
60 Identifying a translationally appropriate animal model for these studies should take into account
61 the placentation type and mechanisms of passive antibody transfer, as well as the transfer of
62 antibodies through breast milk. There are three main types of placentation in mammals based
63 on the tissue types and layers at the materno-fetal interface, including hemochorial (primates,
64 rodents and rabbits), endotheliochorial (carnivores), and epitheliochorial (horses, pigs, and
65 ruminants). The hemochorial placenta is the most invasive type, allowing for direct
66 communication between the maternal blood supply and the chorion, or the outermost fetal
67 membrane. Based on the number of trophoblast layers, there are several variations of
68 hemochorial placentation, including the hemomonochorial placenta found in primates, the
69 hemodichorial placenta in rabbits, and the hemotrichorial placenta observed in rats and mice[7].
70 This direct contact between maternal blood supply and chorion allows for the passive transfer of
71 antibodies across the placenta during gestation. IgG is the only antibody class that significantly
72 crosses the human placenta[8], whereas IgA is the predominant class of Ig found in human breast
73 milk[9]. Of the scientifically relevant models, only primates (including humans), rabbits, and guinea
74 pigs transfer IgG *in utero* and IgA in the milk[10,11]. Therefore, the rabbit model incorporates factors
75 comparable to those in humans that control transplacental transfer of IgG and lactational
76 transfer of IgA.
77
78 In addition to serving as an exceptional model for maternal immunity and vaccine development,
79 similarities between the rabbit and human nasal cavities make them an appropriate model for
80 intranasal immunization. The volume of the rabbit nasal cavity is more similar to humans than
81 rodent models based on relative body mass[12]. Additionally, Casteleyn et al. [12] demonstrated that
82 the nasal associated lymphoid tissue (NALT) is more voluminous in the rabbit compared to
83 rodents. The NALT is located primarily at the ventral and ventromedial aspect of the ventral nasal
84 meatus and at the lateral and dorsolateral aspect of the nasopharyngeal meatus in rabbits,
85 whereas in rodents, the lymphoid tissue is only present along the ventral aspect of the
86 nasopharyngeal meatus[12]. In rabbits, the structure and location of the intraepithelial and lamina
87 propria lymphocytes, as well as the isolated lymphoid follicles, are similar to humans[12].
88

89 Additional advantages of using the rabbit as a model for maternal and mucosal immunity include
90 their high fecundity and relatively short gestation period. Large auricular blood vessels allow for
91 relatively easy access to large volumes of blood for serial collections. A variety of mucosal
92 samples can be collected for antigen-specific antibody response assays, including breast milk[13]
93 (when lactating), mucosal secretions or washes (e.g., oral[14-16], bronchoalveolar lavage[13,17-19],
94 vaginal[20-22]), and feces[20,23-25]. Milk samples can be easily collected during lactation to assess the
95 presence of antigen-specific antibody responses. Though not as abundant as for humans and
96 mice, a wide variety of experimental reagents are available for rabbit-specific studies and assays.
97 In this article, we will describe and demonstrate intranasal immunization and milk collection in
98 New Zealand White rabbits (*Oryctolagus cuniculus*).
99
100 **PROTOCOL:**
101
102 All procedures were approved and performed in accordance with the Duke University IACUC
103 policies.
104
105 NOTE: Materials needed are provided in the **Table of Materials**.
106
107 **1.   Rabbit Sedation and Anesthesia**
108
109 1.1.   Sedate the female rabbit (sexually mature; approximately 5-30 months old) by
110 administering acepromazine intramuscularly (IM) at a dose of 1 mg/kg. Depending on the size
111 of the animal, use a 1 or 3 mL syringe with a 25 G needle. Epaxial muscles are the preferred site
112 of the intramuscular injection.
113
114 NOTE: Acepromazine can also be administered subcutaneously, but IM is preferred by the lab,
115 as it acts more rapidly and reduces the incidence of skin lesions.
116
117 1.2.   Wait 10-15 minutes to allow the acepromazine to take effect.
118
119 1.3.   Anesthetize the rabbit with isoflurane by placing the connected nose cone over the
120 animal's nose. Adjust the vaporizer to up to 4% isoflurane combined with up to 4 liters/minute
121 oxygen. Rabbits have a high aversion to isoflurane, so adequate restraint is necessary when
122 masking the animal.
123
124 1.4.   Once fully anesthetized, as assessed by the pinna, pedal, and/or palpebral reflex, apply
125 ophthalmic lubricant to each eye to prevent drying of the eyes and subsequent corneal
126 ulceration.
127
128 1.5.   Continually monitor reflexes and breathing during anesthesia, and reduce the isoflurane
129 rate to 1-2% once an adequate plane of anesthesia has been reached.
130
131 **2.   Intranasal immunization**
132

133   2.1.   Prepare immunization solution prior to animal handling.

134

135   2.2.   Sedate the rabbit as described above.

136

137   2.3.   Once the lab member is ready to administer the vaccine and the rabbit is in an adequate
138   plane of anesthesia, turn off the isoflurane and oxygen and remove the nose cone.

139

140   2.4.   Place the rabbit in dorsal recumbency, and prop the neck and head at an approximate
141   45° angle that allows easy access and visualization of both nares by the lab member
142   administering the vaccine.

143

144   2.5.   Load the pipette with no more than 100 µL of the vaccine solution, and quickly
145   administer the solution in each nostril. The pipette should be held at an approximate 45° angle,
146   angled towards the medial aspect of the nasal passage.

147

148   NOTE: The goal of immunization is for the solution to contact the mucosal membrane of the
149   nares, so the tip should not be placed within the nares, as this may result in abrasion or
150   irritation of the mucosal tissues and potentially influence the immunogenicity of the nasally-
151   administered vaccine. The vaccine should be administered quickly and done in the same
152   manner in the other nare.

153

154   2.6.   Following administration in both nasal passages, maintain the rabbit in dorsal
155   recumbency for 30 seconds to minimize leakage of the vaccine solution.

156

157   NOTE: The lab will typically administer no more than 100 µL per nostril at a time. If a larger
158   volume is to be administered, with a maximum total of 500 µL, the vaccine can be given in 100
159   µL aliquots with a 30 second rest period between immunizations, and additional
160   administrations of vaccine repeated, with 30 seconds of rest between each administration, until
161   the total vaccine volume is delivered.

162

163   2.7.   Following immunization, place the rabbit on the ventrum for recovery and closely
164   monitor the animal until it can maintain sternal recumbency.

165

166   **3.**   **Milk collection**

167

168   3.1.   Sedate the lactating rabbit as described above.

169

170   3.2.   Clean the skin over the marginal ear vein with the alcohol swab/wipe.

171

172   3.3.   Using a 1 mL syringe and 25 g needle, administer approximately 1-2 IU of oxytocin
173   intravenously via the marginal ear vein to induce milk letdown.

174

175   NOTE: Due to the smooth muscle relaxation, it is common for the rabbit to urinate or defecate
176   following administration of oxytocin.

177
178 3.4.   Following oxytocin administration, apply pressure to the injection site with the piece of
179 gauze.
180
181 3.5.   While maintaining the anesthesia mask over the rabbit's nose, prop the rabbit on its
182 hindquarters.
183
184 NOTE: Milk collection can also be performed with the animal in lateral recumbency, but the lab
185 finds that collection is easier when the rabbit is propped up on its rump with an assistant
186 holding the rabbit upright with the anesthesia mask.
187
188 3.6.   Open the sterile tube to prepare for milk collection and locate the mammary tissue and
189 associated teats. The teats are typically surrounded by wet fur from recent nursing, and the
190 mammary tissue is easily palpable when full of milk.
191
192 3.7.   Grasp the mammary tissue associated with a teat between the thumb and forefinger
193 and apply a gentle, massaging pressure to the glandular tissue in the direction of the teat. Place
194 the collection tube over the teat to collect the expressed milk.
195
196 NOTE: It can sometimes take several minutes for the oxytocin to be effective, and milk
197 production appears to vary among mammary glands. If milk expression is not successful, wait
198 several minutes or rotate around to the additional mammary glands. Milk from all teats can be
199 collected in the same vial. Typically, several milliliters of milk can be easily collected from a
200 lactating doe.
201
202 3.8.   Following milk collection, turn the isoflurane and oxygen off, and allow the rabbit to
203 recover while being closely monitored until the animal is able to maintain sternal recumbency.
204
205 **REPRESENTATIVE RESULTS:**
206 An overview of a typical maternal intranasal immunization study design is depicted in **Figure 1**,
207 incorporating the immunizations, breeding, kindling, lactation, and antibody transfer. Though not
208 illustrated, blood should be collected prior to the initial immunization for baseline measurements
209 and throughout the remainder of the study at regular intervals. Blood is easily obtained via the
210 central ear artery with mild sedation and a topical analgesic agent (e.g., lidocaine 2.5% and
211 prilocaine 2.5% cream). The presence of antigen-specific IgG levels can be measured in these
212 samples. Female rabbits are immunized via the intranasal route, as described in the protocol and
213 demonstrated in the video. Depending on the study, the vaccine may require a boost or may
214 need to be given through an additional route (e.g., intramuscular or subcutaneous). Following
215 study initiation, rabbits are bred; we prefer to purchase proven breeders from vendors to use to
216 ensure a higher pregnancy rate for these studies. Depending on the immunization timeline,
217 rabbits may receive additional immunizations throughout pregnancy. Antigen-specific IgG is
218 transferred transplacentally to the kits, and at approximately 30-32 days post-breeding, pregnant
219 does will kindle. We recommend limiting handling of kits for the first several days to minimize
220 rejection from the does. Blood samples can be collected from the kits to assess antigen-specific

IgG levels that were transferred transplacentally (**Figure 3**). In addition to a wide variety of nutrients, kits receive IgA from the lactating doe while nursing. Kits are typically weaned at 4-8 weeks, but prior to weaning, milk can easily be collected from lactating does, as demonstrated in the video. The collected milk samples can then be processed for the detection of total and antigen-specific IgA levels (**Figure 4**). Depending on the study, vaccines (+/- boosts) can be administered to the kits, and serial blood samples can be collected from the kits at a very early age using the lateral saphenous vein.

For maternal studies, determining pregnancy as early as possible is helpful for the study design and for ensuring the doe does not need to be rebred. Progesterone measurements can be used as a means to detect pregnancy. As shown in **Figure 2**, elevated progesterone levels can be detected in pregnant rabbits compared to non-pregnant rabbits even after matings by a buck were confirmed for all does. There are additional methods for pregnancy detection, including manual palpation, ultrasound, and radiographs; however, these require well-trained personal and proper equipment.

Antigen-specific IgG that was transferred transplacentally while *in utero* can be measured in the serum of kits. Blood can be collected from a small number of kits at or near the time of birth to assess early antigen-specific antibody levels, but serial blood collection is technically much easier as the kits age and increase is size. As depicted in **Figure 3**, serum levels of antigen-specific IgG in the kits can be measured by ELISA and compared to the maternal levels. Maternally transferred IgG levels tend to be higher at birth and decrease over time.

As a type of mucosal sample, milk can be collected and processed to measure total or antigen-specific antibody levels. As shown in **Figure 4**, IgA makes up a significant portion of the total antibody levels within the breast milk that is being transferred to the kits via lactation. Our results demonstrate that breast milk IgA produces a slightly higher ELISA signal (relative light units, RLU) when compared to IgG, and both IgA and IgG produce a signal that is much higher than the signal for IgM. These results are in agreement with results from others that suggest that rabbit milk contains around 4.5 mg/mL IgA, 2.4 mg/mL IgG, and 0.1 mg/mL IgM[26,27].

FIGURE AND TABLE LEGENDS:
Figure 1. Sample timeline for a maternal intranasal immunization study design in a rabbit (*Oryctolagus cuniculus*) model. Female rabbits are immunized via the intranasal route, as described in the protocol and demonstrated in the video. Depending on the study, the vaccine may require a boost or may need to be given through an additional route (e.g. intramuscular or subcutaneous). Rabbits are then bred. Antigen-specific IgG is transferred transplacentally to the kits, and at approximately 30-32 days post-breeding, pregnant does will kindle. IgA is passed to the kits from the lactating doe while nursing. Prior to weaning, milk can easily be collected from the lactating does to assess total and antigen-specific IgA levels.

Figure 2. Progesterone levels in pregnant and non-pregnant rabbits at 3 weeks post-breeding. Blood was collected from rabbits at 3 weeks post-breeding. Rabbits were confirmed either pregnant or non-pregnant based on ability to kindle a litter at 30-32 days post-breeding. Serum

265 progesterone levels were measured through the Michigan State University Veterinary Diagnostic
266 Laboratory using a chemiluminescent immunoassay (CLIA) with an immunoassay system (e.g.,
267 Siemens Healthineers IMMULITE 2000). Error bars represent standard error of the mean, and the
268 sample size consisted of 4-6 rabbits per group.
269
270 **Figure 3. Antigen-specific IgG levels in kits (relative to maternal levels) at birth and at 3 weeks**
271 **of age following a series of maternal immunizations.** Blood was collected from the does and kits
272 soon after kindling and at 3 weeks of age. Antigen-specific IgG levels within the serum were
273 detected using a fluorescent ELISA as previously described[28]. Antigen-specific IgG is plotted as a
274 ratio of levels detected in the kit serum and maternal serum.
275
276 **Figure 4. A comparison of IgA, IgM, and IgG levels in rabbit milk.** Rabbit milk was collected as
277 described and demonstrated in the video. Milk was processed by a long centrifugation (13,000 x
278 g for 4.5 hours at 4 °C), and the clear middle layer was isolated following processing. Total IgA,
279 IgG, and IgM levels were measured in this clear layer by fluorescent ELISA as previously
280 described[28], except that plates were coated with polyclonal anti-IgA, anti-IgG, or anti-IgM to
281 detect total rabbit IgA, IgG, or IgM, respectively.
282
283 **DISCUSSION:**
284 Although not described in the above protocol, successful breeding of the rabbits is necessary for
285 this maternal model and to allow for milk collection. Rabbits are easily bred by live cover in a
286 research setting. It is recommended that does be transferred to the buck's cage for breeding, as
287 does can be territorial and aggressive if kept in their own cage with the buck. If females are non-
288 receptive after 15 minutes (as indicated by running away biting, or vocalizing), the doe should be
289 placed back into her own cage. There are several informative videos and tutorials on rabbit
290 breeding that can be observed online[29], but after breeding, the male will typically fall over and
291 may vocalize. Once this is observed, the doe can be returned to her cage. A buck may breed 2-3
292 times a day with no decrease in sperm count[30]. In our IACUC-approved protocol, bucks are limited
293 to breeding 10-12 does per week and are provided at least two rest days per week, as sources
294 state that a single buck is usually sufficient to service 10-15 does[31]. Our group suggests purchasing
295 proven breeders from the vendor to improve the breeding success rate. As rabbits are induced
296 ovulators and ovulation typically occurs 10-13 hours post-copulation[31], we have experienced a
297 higher rate of pregnancy in does that are bred in the morning and then again in the afternoon,
298 or twice within that 10-13 hour window (observed increase from 75% to 95% success rate,
299 unpublished). Based on the literature, typical breeding success rates vary from 57-100%[32-40], and
300 litter sizes average approximately 7-9 kits[31].
301
302 Determining pregnancy as early as possible is helpful in maternal studies to confirm that the doe
303 does not need to be rebred or removed from the study. Options for pregnancy detection include
304 palpation (as early as 14 days)[31], ultrasound (as early as 5-9 days)[40], radiographs (as early as day
305 11)[31], weight gain, and molecular techniques, such as measurements of insulin-growth factors[41]
306 (IGF) and progesterone[34,37,38,42,43]. Previous work has indicated significant elevations of IGF-II
307 levels in pregnant rabbits compared to levels in non-pregnant rabbits[41]. However, in our hands,
308 we were unable to detect a difference in IGF-II levels between pregnant and non-pregnant rabbits

309   (unpublished). As adequate progesterone levels are necessary for the maintenance of pregnancy
310   in rabbits[37,44], several studies have assessed progesterone levels in pregnant rabbits and
311   demonstrated elevated levels relative to non-pregnant rabbits, particularly during organogenesis
312   around mid-gestation[34,43,44]. Our group was unable to detect differences in progesterone levels
313   using an ELISA between pregnant and non-pregnant rabbits, but preliminary results using an
314   automated chemiluminescent assay at the Michigan State University Veterinary Diagnostic
315   Laboratory indicate elevated progesterone levels in pregnant rabbits compared to non-pregnant
316   rabbits even after matings by a buck were confirmed for all does assessed (**Figure 2**).
317
318   Lactating does can produce approximately 250 mL, or 60 mL/kg, of milk daily[45,46], allowing for
319   large volumes for experimental assays to assess total and antigen-specific antibody
320   responses/concentrations. Rabbit milk contains high levels of fat and protein, containing 2 and 3
321   times more concentrated levels of fat and protein, as compared to cow and sow milk,
322   respectively[45,47]. Due to the high fat content in the milk, the samples require significant
323   processing, depending on the assays to be conducted. Following centrifugation of the milk
324   sample, three distinct layers are separated out, including the cells within the bottom layer, clear
325   intermediate layer containing the immunoglobulins, and fat within the top layer[48].
326   Immunoglobulins, within the clear intermediate layer, are present at high concentrations within
327   the colostrum and milk and are primarily made up of IgA, IgG, and IgM (**Figure 4**). Though milk
328   samples are easily collected by hand, and this is our preferred technique within the lab, vacuum
329   systems have also been reported in the literature[13,46,48]. Yoshiyama et al.[48] collected milk samples
330   using negative pressure and described a long centrifugation (15,000 x g for 4 hours) for separation
331   of the milk layers prior to passing the clear intermediate layer through a Sepharose 4B column
332   for immunoglobulin removal. Using this method, authors were able to detect cholera toxin-
333   specific antibodies within the rabbit milk of orally immunized rabbits at sufficient levels for
334   protection against *Vibrio cholerae*-induced secretion in the intestine[48]. Peri et al.[13] processed milk
335   samples collected by aspiration with a water vacuum system by centrifugation at 4 °C for 2 hours
336   at 24,000 x g. In this study, authors were able to detect anti-RSV IgA in the colostrum, milk, and
337   bronchial and intestinal secretions following mucosal immunization (oral or intratracheal), but
338   not intravenous immunization, whereas anti-RSV IgG was detected in colostrum, milk and serum
339   regardless of immunization route[13].
340
341   Care should be taken during the intranasal immunization procedure to confirm the rabbit is in an
342   adequate plane of anesthesia and to avoid administering large volumes of vaccine at once.
343   Previous work from our group has shown that the efficacy of the intranasal immunization was
344   influenced by the use of anesthesia[49]. Specifically, deep anesthesia induced by the combination
345   of ketamine and xylazine correlated with increased immunogenicity of the nasally administered
346   vaccine and improved nasal retention of the solution. These levels were significantly higher than
347   retention and immunogenicity in rabbits intranasally immunized following sedation with
348   acepromazine and butorphanol[49]. Similar findings have also been demonstrated following
349   intranasal immunization in mice[50,51]. Additionally, our work demonstrated increased
350   immunogenicity following intranasal immunization under the deep anesthesia over animals that
351   were fully awake and over animals undergoing shorter duration anesthesia with a combination
352   of acepromazine and isoflurane. This difference in IgG immunogenicity between deep versus

353 shorter duration anesthesia was significant at the 42 day time point but not at the 56 day time
354 point. Although increased immunogenicity and retention may result from the deeper anesthesia,
355 rabbits required at least 30 minutes to recover; whereas, rabbits were upright and mobile within
356 5 minutes of receiving the nasal vaccine following the shorter duration isoflurane-induced
357 anesthesia. Although anesthesia may not be ideal for mimicking the clinical setting for intranasal
358 immunization in humans, a shorter duration anesthesia (e.g., isoflurane) may be preferred to a
359 longer, deep anesthesia with injectable agents (e.g., ketamine + xylazine). The use of an animal
360 model, as demonstrated in Gwinn et al.[49], will often require the use of sedation or anesthesia to
361 allow for safe animal handling and effective and consistent vaccine delivery. A potential limitation
362 for anesthesia use with vaccine administration in animal models is its effect on the ability of the
363 animal to induce an appropriate immune response to the vaccine. Interestingly, reports in the
364 literature suggest that isoflurane can attenuate systemic inflammatory responses, providing
365 protection against challenges[52] and reducing oxidative stress and inflammation[53].
366
367 With respect to minimization of vaccine loss and adequate intranasal delivery, our group
368 recommends a rest period of 30 seconds after administering each aliquot of the intranasal
369 vaccine to allow for retention of the solution within the nasal cavity and to prevent the solution
370 from seeping out from the nostrils if the rabbit is being immediately placed in sternal recumbency
371 and returned to the cage. Additionally, it is important to limit the volume of the aliquot
372 administered to ensure intranasal immunization, as mucosal absorption is limited by the surface
373 area of the nasal passage. If a large volume is administered, the solution may bypass the nasal
374 mucosa and result in inhalational or gastric immunization. Our group recommends aliquots of
375 100 µL per nostril with a maximal intranasal volume of 500 µL.
376
377 This article focuses on mucosal vaccine delivery, specifically via the intranasal route, but there
378 are multiple methods of immunization, both mucosal and parenteral that have been
379 demonstrated in rabbits. These additional methods include, but are not limited to, oral,
380 subcutaneous, intramuscular, and intradermal. As such, samples to be collected and assessed
381 vary based on the experimental goals and route of immunization.
382

392

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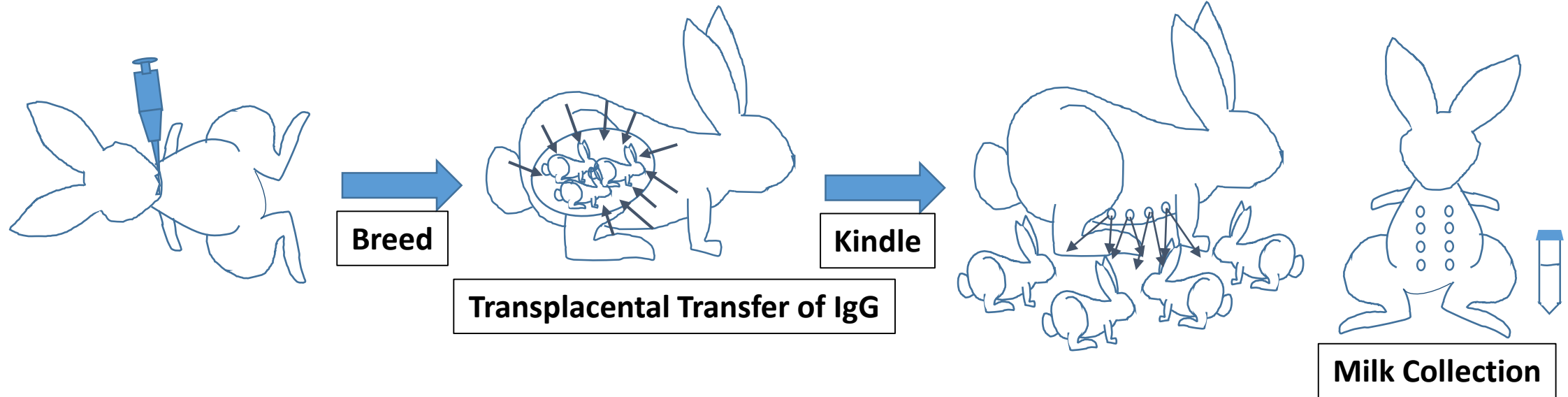526 50 Sloat, B. R., Cui, Z. Evaluation of the immune response induced by a nasal anthrax vaccine
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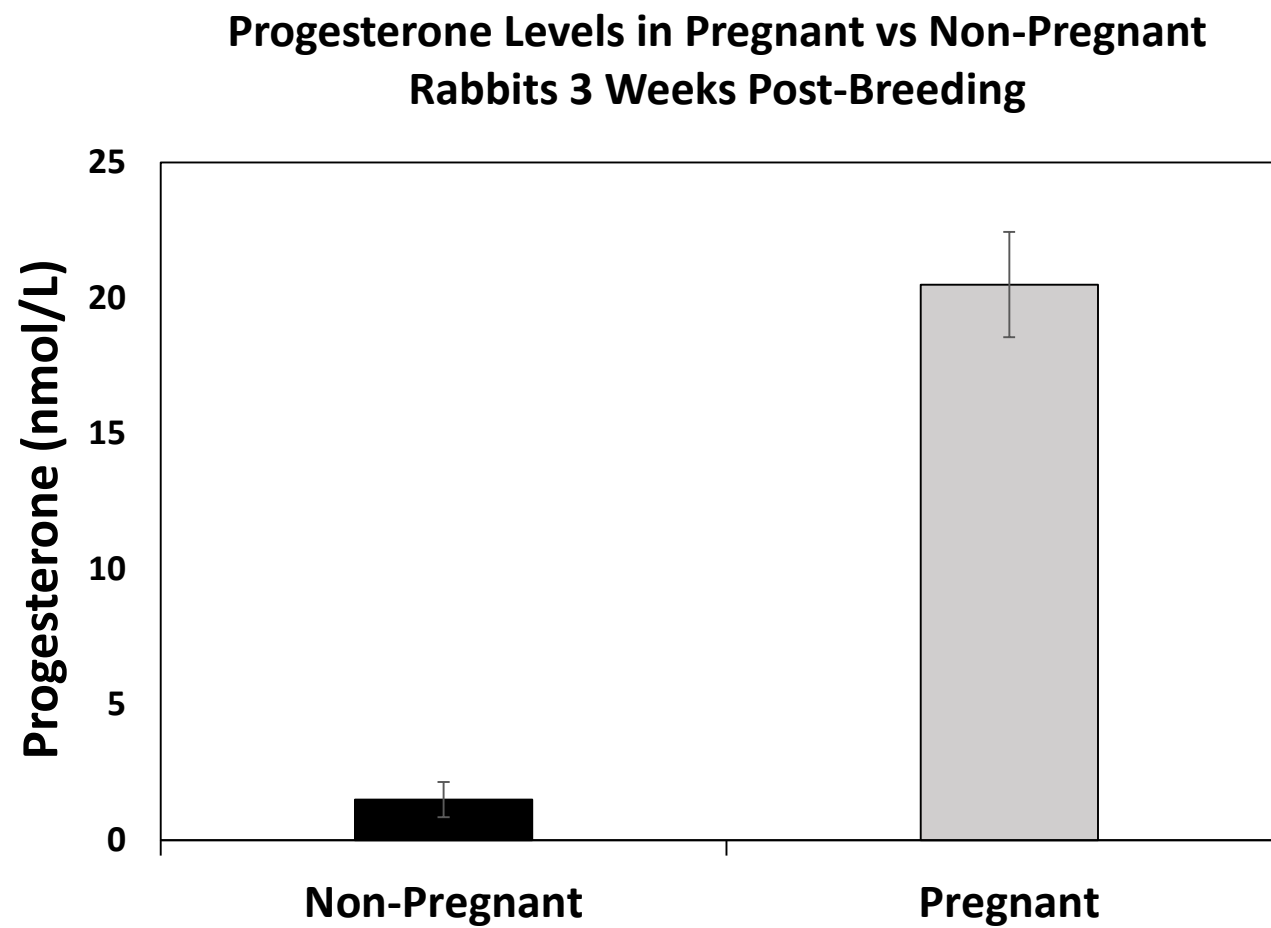
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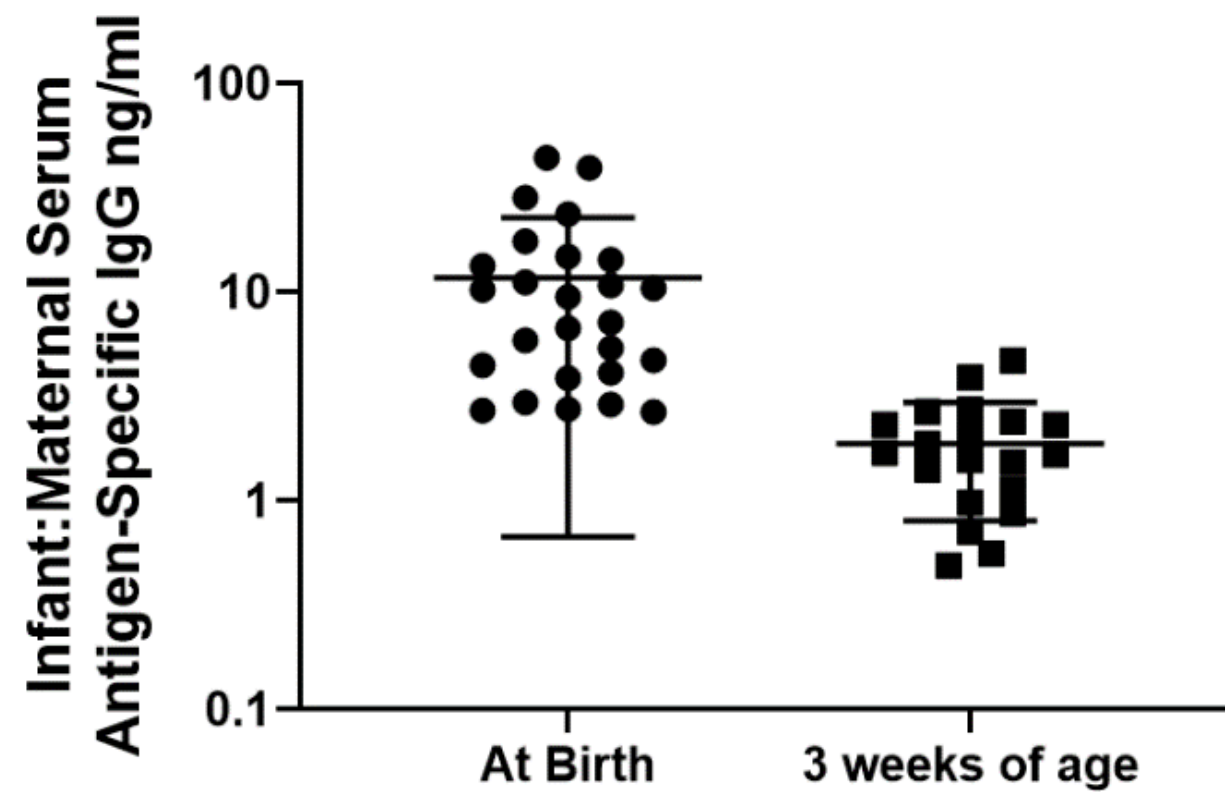535

Figure 1

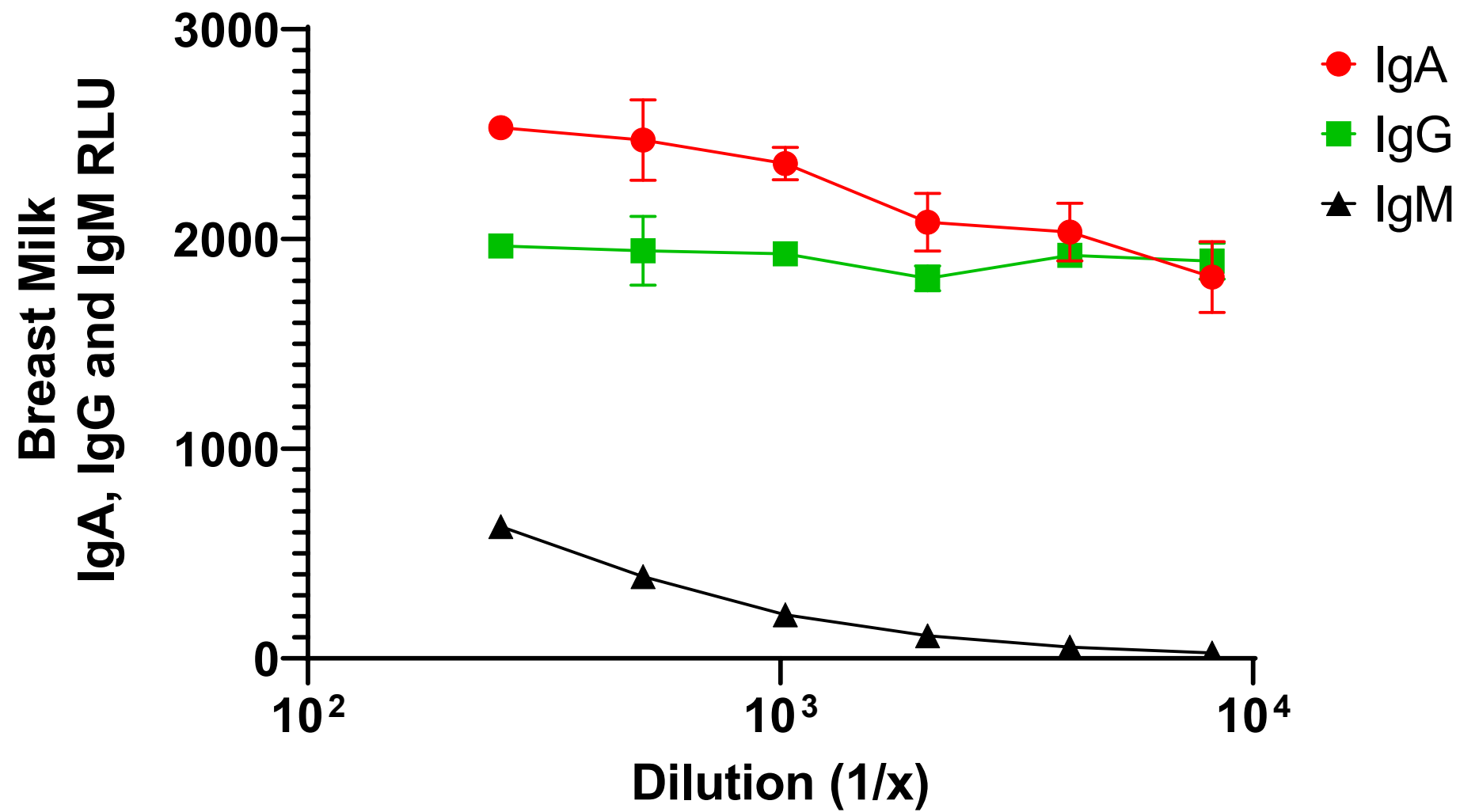# Maternal Intranasal Immunization Study Design

Intranasal Immunization +/- Boost

Lactational Transfer of IgA

Breed

Transplacental Transfer of IgG

Kindle

Milk Collection

Figure 2

Progesterone Levels in Pregnant vs Non-Pregnant Rabbits 3 Weeks Post-Breeding

Figure 3

Figure 4

Intranasal Immunization

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Anesthesia Machine/Vaporizer	Vet Equip	901807	
Hypodermic Needle (25 g)	Terumo	07-806-7584	
Isoflurane (250 mL Bottle)	Patterson Veterinary	07-893-1389	2-4%
Luer Lock Syringe (1 mL)	Air-Tite	07-892-7410	
Mucosal Vaccine	N/A	N/A	Experimental Vaccine
Nose Cone	McCulloch Medical	07891-1097	
Pipette Tips	VWR	53503-290	
Pipettor	VWR	89079-962	
PromAce (Acepromazine maleate)	Boehringer Ingelheim	07-893-5734	1mg/kg IM
Puralube Sterile Ophthalmic Ointment	Dechra	07-888-2572	

Milk Collection

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Alcohol Prep 2-ply	Covidien	07-839-8871	
Anesthesia Machine/Vaporizer	Vet Equip	901807	
Hypodermic Needles (25 g)	Terumo	07-806-7584	
Isoflurane (250 mL Bottle)	Patterson Veterinary	07-893-1389	2-4%
Luer Lock Syringe (1 mL)	Air-Tite	07-892-7410	
Non-Woven Sponge (4x4)	Covidien	07-891-5815	
Nose Cone	McCulloch Medical	07891-1097	
PromAce (Acepromazine Maleate)	Boehringer Ingelheim	07-893-5734	1mg/kg IM
Puralube Sterile Ophthalmic Ointment	Dechra	07-888-2572	
Sterile Conical Vial (15 mL)	Falcon	14-959-49B	

U **Duke** University School of Medicine

Herman F. Staats, Ph.D.
Professor and Vice Chair for Research
Department of Pathology

May 7, 2021

Nam Nguyen, Ph.D.
Manager of Review
JoVE
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617.674.1888

Dear Dr. Nguyen,

On behalf of the authors, I would like to thank the reviewers for their helpful comments for our manuscript, JoVE62317 "Intranasal immunization and milk collection in studies of maternal immunization in New Zealand White rabbits (*Oryctolagus cuniculus*)". Their comments were very valuable, and we have attempted to address their comments.

We hope the revised manuscript addresses the reviewers' concerns and is now acceptable for publication.

Please contact me if you require any additional information.

Sincerely,

Herman F. Staats, Ph.D.
Professor of Pathology

Editorial comments:

Changes to be made by the Author(s) regarding the <u>written manuscript</u>:
1. Please remove the Materials needed sections in the protocol and cite the Table of Materials instead.

Response: The list of materials has been removed, and the Table of Materials has been referenced.

Changes to be made by the Author(s) regarding the <u>video</u>:
1. Please reflect the title from the written manuscript in the title card of the video as well.

Response: The title has been changed in the video to reflect the manuscript title.

2. Please increase the homogeneity between the written protocol and the video narration. Ideally, the narration is a word for word reading of the written protocol so users can follow along.

Response: The narration has been modified to be more similar to the written protocol (only minor changes incorporated to help with viewer understanding).

3. Authors and institutions have to be with the main title in the beginning of the video, not at the end of the introduction

Response: The authors and institutions have been moved to the beginning of the video near the title slide.

4. Turn the speaker audio up a little bit

Response: The speaker volume has been increased.

5. Please have a protocol title card.

Response: A protocol title card/slide has been incorporated. Thank you for the clarification.

6. The video is too long, find spots to cut down. For example, you don't need to show the full 30 second countdown between doses at 5:04, or show the full materials lists at 3:17 and 6:19. Cutting down the milking section at 8:10 will also help with time.

Response: The video has been shortened based on the recommendations.

7. Please only show the animal use statement once at the beginning of the protocol.

Response: The animal use statement is now only showed once prior to the protocol.

Please upload a revised high-resolution file here:
https://www.dropbox.com/request/6dYRJb51nfxJ7Y6gJJYx?oref=e

Vet Review
Yes, animals were used humanely and appropriate anesthesia and analgesia were administered. I have

no corrections or suggestions.
Resolved.

Reviewer #2
Overall video is pretty well organized and presentation is well done.

My suggestions are dependent to some extent on what the audience is for this video.

1. I have concerns of naive people not understanding how to use acepromazine for example, that it is injected, or why it is given (to sedate rabbit to get it hooked up to mask for isoflurane for full anesthesia). So in slides of materials needed - i suggest adding a bit more info - injection or administration method, and why it is needed (acepromazine, for sedation prior to anesthesia). the material slides are at 3.24 and 6:20 into the presentation.
    Response: There is an anesthesia protocol provided in the manuscript that describes this process. We wanted to emphasize the immunization and milk collection in the video, so we limited the protocol to these methods. The descriptions, as suggested, have been added to the materials slides.
2. also they list cone (need to add for anesthesia which is not listed), but in oral presentation at 6:40 they refer to mask instead of cone. terminology should be consistent.
    Response: The terminology has been modified to be more consistent.
3. I think it would be helpful to show how mask and machine are connected to rabbit at some point.
    Response: Based on the video criteria, we thought that shots and/or visual depictions of euthanasia or anesthesia were not allowed.
4. Can an alternative gas be used other than isoflurane? again this is for naive people doing this work.
    Response: Other inhalant anesthetics such as sevoflurane could be used for anesthesia; however, we wanted to reflect the procedures performed by our lab currently and historically. See Gwinn, W. M. *et al.* Effective induction of protective systemic immunity with nasally administered vaccines adjuvanted with IL-1. *Vaccine.* **28** (42), 6901-6914, (2010).
5. On material slides for lactation gauze and alcohol are listed but not included in discussion what they are for.
    Response: This is now addressed in the narration.
6. For lactation slide of materials they list 15 mL conical tube - it may be useful to include what type of plastic should be used since proteins and fats may bind to plastic (polystyrene vs polypropylene or polycarbonate, for example). which could be critical for harvesting antibodies.
    Response: The conical tube ordering information is provided in the materials table, but the tubes we typically used are made of polypropylene. Though there are different binding characteristics across the various plastics, milk has extremely high concentrations of antibodies, proteins, and fats, decreasing our concern for non-specific binding and harvesting the antibodies of interest.
7. The other inconsistent terminology is at 9:30 they discuss grams of milk but later at 12:00 they refer to milk volume in mL. i think mL would be more useful term. if grams are needed they should explain why.

Response: This information has been has been removed from the narration and is clarified in the manuscript.

8. At 13:00 they talk about methods to determine pregnancy but slide shows progesterone levels at 3 wk post breeding. Since gestation of rabbit is 31-32 days, this is pretty far into the pregnancy before it is confirmed. seems like there should be a method (ultrasound or x/ray) that can determine pregnancy much earlier than 3 weeks.
   Response: Various techniques and the timing for determining pregnancy are included in the manuscript. Though we measured progesterone levels at 3 weeks, progesterone measurements can be performed earlier, around mid-gestation. The benefit to the progesterone measurement is that it is technically easy, not requiring specialize equipment (eg US or X-ray machine) or experienced staff (eg for imaging or palpation).

9. 10:00 - 11:00 there is dead air time as they show collection of milk from rabbit. This should be shorter and number of times or total time needed for milk collection should be discussed and less time used on video. In first half of presentation the vaccination timing could be reduced as well.
   Response: This portion of the video has been shortened. Thank you for the recommendation.

10. 10:30 they discuss alternative method to induce lactation chemically without breeding. This seems a bit out of place here and perhaps a reference to how this is done would be better.
    Response: This portion of the narration has been removed and video section shortened.

11. in the vaccination phase they discuss and show placing rabbit in dorsal recumbency to recover which is important so they don't aspirate ingesta. This should be repeated for milk collection as well.
    Response: The recovery procedures are stated in the narration and demonstrated following intranasal immunization, but as we were asked to reduce the length of the video, we did not think this demonstration needed to be repeated.

12. timing of when to take the rabbit off anesthesia was mentioned in the vaccination phase (but could be dependent on length of time needed for vaccination). but was not discussed in lactation phase. of course this would vary as milk collection takes much more time but should be addressed.
    Response: This information has been added to the narration.