• Basic Research •

# Transfer of single dose of intravitreal injection of ranibizumab and bevacizumab into milk of sheep

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## Abstract

**• AIM: To investigate whether single-dose intravitreal injections of bevacizumab and ranibizumab transfer into milk.**

**• METHODS: This study included lactating 12 sheep and a single 3-month old suckling lamb of each sheep. Two groups consisting of 6 sheep and their lambs were constituted; the ranibizumab group and the bevacizumab group before the administration of intravitreal injections, blood and milk samples were obtained from all sheep and, following the injections, blood and milk samples of all sheep and blood samples of all lambs were collected at regular time points. Serum and milk concentrations of bevacizumab and ranibizumab were measured using an enzyme-linked immunosorbent assay (ELISA) kit. The limit of determination was 0.9 ng/mL for bevacizumab and 0.62 ng/mL for ranibizumab.**

**• RESULTS: At 6h after intravitreal injections, bevacizumab concentration was above the limit of determination in the blood of all sheep. At 3wk, when the study was terminated, bevacizumab concentrations were high in 4 sheep. Even though bevacizumab concentrations in milk showed fluctuations, the drug transferred into the milk of all sheep at detectable concentrations. Ranibizumab drug concentrations in the blood and milk of sheep and those in the blood of lambs were below the limit of determination by the ELISA kit.**

**• CONCLUSION: This sheep model study demonstrate that intravitreal injection of ranibizumab, which did not transfer into the milk of sheep and suckling lambs, is safer than bevacizumab during lactation period.**

**• KEYWORDS:** bevacizumab; ranibizumab; lactation; milk; vascular endothelial growth factor

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## INTRODUCTION

A literature review has shown that anti-vascular endothelial growth factors (VEGF) are used in the treatment of a wide range of diseases, including senile macular degeneration, diabetic macular edema, retinal vein occlusion, central serous chorioretinopathy, retinitis pigmentosa, choroidal neovascular membranes secondary to degenerative myopia, idiopathic choroidal neovascular membranes, ocular surface diseases and macular edema secondary to inflammations[1-13].

Even though vitreous and blood levels of these drugs and their half lives in these fluids are well known, there is a lack of large-scale and animal studies in the literature on whether these drugs transfer into breast milk and their use in nursing patients and what precautions are to be taken in babies of these mothers.

There is a lack of data in the literature concerning whether it is safe to use these drugs in diseases that may pose a threat on vision, including central serous chorioretinopathy, idiopathic choroidal neovascular membrane and diabetic retinopathy, which are worsened by the effects of increased VEGF and placental growth factor during pregnancy and lactation.

The aim of this study was to identify whether intravitreal injections of ranibizumab and bevacizumab transferred into milk, and if yes, whether they transferred into the blood of suckling animals.

## MATERIALS AND METHODS

**Animals and Plan of Experiment** This study included 12 "pirit" sheep in the lactation period, from 3 to 4y of age, weighing 40-45 kg and a single 3-month old suckling lamb

of each sheep. Animals were given *ad libitum* access to green grass, feed and water. Lambs were kept with their mothers and they were separated only on sampling days for one about hour.

The sheep and their lambs were categorized into two groups; the ranibizumab and the bevacizumab groups, with 6 animals each. Two days before the intravitreal injections, all sheep received 3% ofloxacin eye drops (Exocin, Alcon, Inc., Switzerland) for four times a day as prophylaxis of endophthalmitis. At the end of day 2, an average of 2 mL blood was collected from each sheep before the administration of intravitreal injections. After complete milking of the gland, about 2 mL of milk sample was obtained from each sheep.

All serum samples were centrifuged in the cold at 1500 rpm for 10min, the supernatant was taken into Eppendorf tubes and kept in frozen at -80℃. The milk samples were directly stored in freezer at -80℃.

After aseptic conditions were ensured, animals were immobilized and drops of 0.5% proparacaine hydrochloride (Alcaine, Alcon, Inc., Switzerland) were administered into their eyes and topical anesthesia was achieved by placing topical anesthetic-impregnated sponges into the fornices. Periocular antisepsis was achieved using 10% povidone iodine and a blepharostat was placed. After the instillation of 5% topical povidone iodine into the fornices, povidone iodine was washed out following a 3-minute waiting period.

One group received intravitreal injection of ranibizumab (0.5 mg/0.05 mL) and the other group received intravitreal injection of bevacizumab (1.25 mg/0.05 mL) in the superior temporal quadrant of the sclera, 3.5 mm posterior to the limbus using a 26G insulin needle. During the withdrawal of the syringe after injection, a cotton-tipped applicator was pressed onto the injection site. The procedure was completed after the application of an antibiotic pomade. The instillation of topical antibiotic drops (Exocin, Alcon, Inc., Switzerland) into the injected eye was continued on a basis of four times a day for a further 5d.

The study was continued for 3wk in the bevacizumab group sheep and 1wk in the ranibizumab group sheep. On the other hand, lambs were followed up for 1d. After intravitreal injections, blood and milk samples were collected from the bevacizumab group at hours 3, 6, 12, 24, on days 2, 3, 5 and at weeks 1, 2, 3, whereas, blood and milk samples were collected from the ranibizumab group at hours 3, 6, 12, 24, on days 2, 3, 5 and at week 1. Blood samples were collected from the lambs in each group at hours 6, 12, and 24. Anterior segment examination was performed in all sheep before the intravitreal injections and on the days of sample collection.

There was few animal study in the literature which try to investigate neither intravitreal injections of ranibizumab and bevacizumab transferred into milk, nor they transferred into the blood of suckling lambs. Drug concentrations in the blood of sheep were below the limit of determination before injection. So we did not collect any sample from lambs at 0 hour. Thus sampling time of suckling lambs started 6$^{th}$ hours of injection.

**Measurements and Evaluation** In accordance with the manufacturer's protocol, bevacizumab and ranibizumab serum and milk concentrations were measured using an enzyme-linked immunosorbent analysis (ELISA) kit (Protein Detector ELISA Kit; KPL, Inc., Gaithersburg, Maryland, USA). Micro plates (Immuno 96 MicroCell solid plates, Nunc, Roskilde, Denmark) were coated with recombinant human $VEGF_{165}$ (RD Systems, Inc., Minneapolis, MN, USA) at a concentration of 1.0 μg/mL at room temperature for one hour (100 μL/well). To prevent nonspecific binding, the wells were blocked with 1% bovine serum albumin/PBS. After then, samples of 100 μL each and standards at different concentrations were added to the plates. Standard curves were generated using bevacizumab and ranibizumab concentrations ranging from 1 ng/mL to 500 ng/mL. There was no problem in standard's absorbance. At the same time, there was no elevated absorbance values in basal samples. These samples were taken before drug administration. The bound bevacizumab and ranibizumab were detected by 0.1 μg/mL of horseradish peroxidase conjugated goat anti-human IgG (H+L) prepared using the ELISA kit. Optic density was read at 405 nm. The limit of determination for bevacizumab and ranibizumab were 0.9 ng/mL and 0.62 ng/mL, respectively. Free bevacizumab and ranibizumab were detected with this experiment and all measurements were performed twice in accordance with manufacturer's instructions[14]. All animal procedures complied with the ARVO Statement for the Use of Animals in Ophthalmic and Visual Research.

**RESULTS**

Tables 1 and 2 present drug concentrations in the blood and milk of the bevacizumab group sheep, the Figures 1 and 2 show changes in drug concentrations over time, Table 3 presents drug concentrations in the blood of lambs and the Figure 3 show changes over time. In this group, the bevacizumab concentrations in the blood of all sheep were above the limit of determination at 6h after the intravitreal injection. Maximum concentrations of bevacizumab were reached at the 48$^{th}$ hour in sheep 3 and at the 72$^{th}$ hour in the remaining sheep. At week 3, when the study was terminated, bevacizumab concentrations were still above the limit of determination in 4 sheep. The bevacizumab concentration in milk showed fluctuations among sheep and even in the same sheep. On the other hand, it was found that bevacizumab transferred into the milk of all sheep at detectable concentrations. Bevacizumab was at detectable levels for 24h starting from hour 6 in suckling lambs. The highest level of concentration in the suckling lamb 4 plasma was 15.22 ng/mL at hour 24.

In the ranibizumab group, drug concentrations in the blood and milk of sheep and those in the blood of lambs were below the limit of determination of 0.62 ng/mL by the ELISA kit.

**Table 1 Drug concentrations in the blood of sheep receiving the intravitreal injection of bevacizumab** (ng/mL)

| Sheep/time | 0h | 3h | 6h | 12h | 24h | 48h | 72h | 5d | 7d | 2wk | 3wk |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Sheep 1 | <0.9 | <0.9 | 11.30 | 15.22 | 49.91 | 87.48 | 106.31 | 91.39 | 94.78 | 86.96 | 76.00 |
| Sheep 2 | <0.9 | <0.9 | 9.13 | 7.97 | 12.87 | 19.65 | 54.61 | 9.42 | 8.84 | 3.03 | 2.74 |
| Sheep 3 | <0.9 | 14.96 | 9.13 | 8.84 | 9.42 | 35.56 | 23.83 | 16.26 | 9.42 | 1.87 | <0.9 |
| Sheep 4 | <0.9 | 10.26 | 12.35 | 6.22 | 5.64 | 78.35 | 11.61 | 24.35 | 6.18 | 5.06 | 3.61 |
| Sheep 5 | <0.9 | 9.71 | 11.83 | 17.04 | 18.87 | 72.09 | 99.74 | 89.57 | 74.70 | 78.87 | 68.23 |
| Sheep 6 | <0.9 | 8.26 | 6.81 | 9.13 | 11.56 | 27.22 | 28.78 | 8.55 | <0.9 | <0.9 | <0.9 |

**Table 2 Drug concentrations in the milk of sheep receiving the intravitreal injection of bevacizumab** (ng/mL)

| Sheep/time | 0h | 3h | 6h | 12h | 24h | 48h | 72h | 5d | 7d | 2wk | 3wk |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Sheep 1 | <0.9 | <0.9 | <0.9 | <0.9 | <0.9 | 0.91 | 28.33 | 2.69 | <0.9 | 14.17 | <0.9 |
| Sheep 2 | <0.9 | <0.9 | <0.9 | <0.9 | 2.69 | 19.20 | 5.50 | 3.25 | 1.00 | <0.9 | 3.81 |
| Sheep 3 | <0.9 | 2.13 | <0.9 | <0.9 | 2.13 | <0.9 | 4.38 | <0.9 | <0.9 | <0.9 | <0.9 |
| Sheep 4 | <0.9 | 0.95 | 2.13 | <0.9 | 1.00 | 0.91 | 30.00 | <0.9 | <0.9 | 1.00 | 4.94 |
| Sheep 5 | <0.9 | <0.9 | <0.9 | <0.9 | <0.9 | <0.9 | <0.9 | 32.50 | <0.9 | 6.06 | 6.63 |
| Sheep 6 | <0.9 | <0.9 | <0.9 | <0.9 | <0.9 | <0.9 | <0.9 | <0.9 | <0.9 | 0.91 | <0.9 |

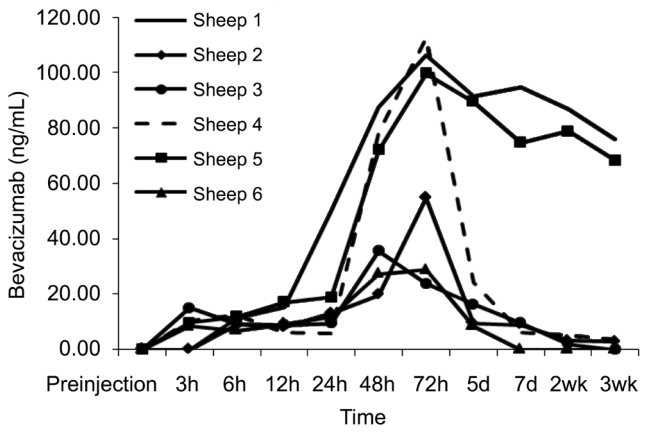


**Figure 1 Drug concentrations in the blood of sheep receiving the intravitreal injection of bevacizumab.**

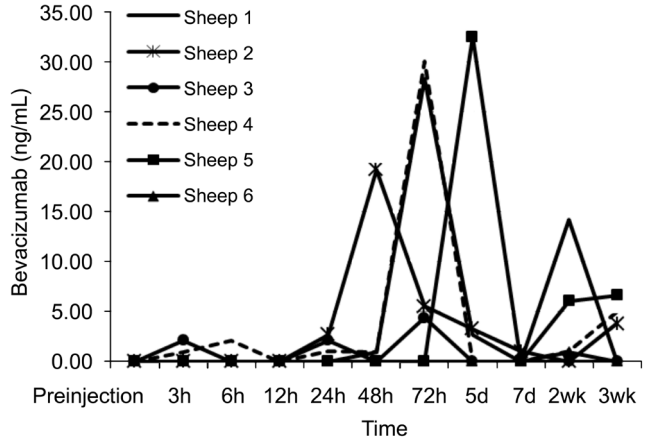


**Figure 2 Drug concentrations in the milk of sheep receiving the intravitreal injection of bevacizumab.**

Three sheep in which ranibizumab was administered and 1 sheep in which bevacizumab was administered had subconjunctival hemorrhage and 1 sheep in the bevacizumab group developed cataract. None of the eyes developed corneal edema, blurriness in the anterior chamber or endophthalmitis.

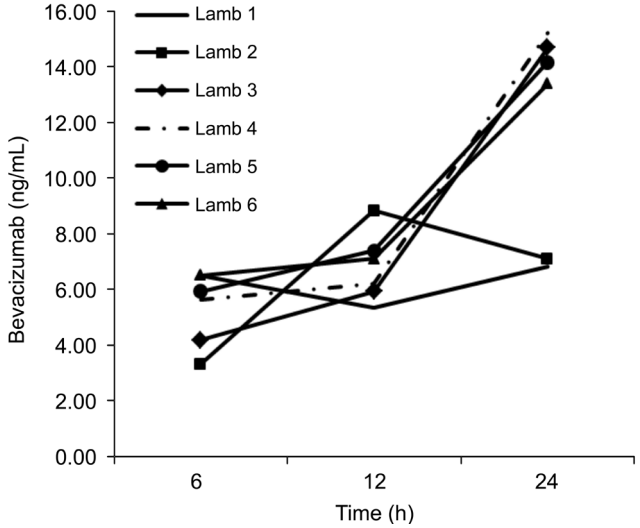


**Figure 3 Drug concentrations in the blood of the suckling lambs of the sheep receiving intravitreal injection of bevacizumab.**

**Table 3 Drug concentrations in the blood of the suckling lambs of the sheep receiving intravitreal injection of bevacizumab** (ng/mL)

| Lamb/time | 6h | 12h | 24h |
|---|---|---|---|
| Lamb 1 | 6.51 | 5.35 | 6.81 |
| Lamb 2 | 3.32 | 8.84 | 7.10 |
| Lamb 3 | 4.19 | 5.93 | 14.69 |
| Lamb 4 | 5.64 | 6.22 | 15.22 |
| Lamb 5 | 5.93 | 7.39 | 14.17 |
| Lamb 6 | 6.51 | 7.10 | 13.39 |

## DISCUSSION

The purpose of this study was to investigate whether intravitreal administration of bevacizumab and ranibizumab transferred into the milk and blood of sheep and the blood of their suckling lambs. Rabbit eyes have been usually used in pharmacokinetic studies of intravitreally administered drugs[15-18] . However, the vitreous volume in a rabbit eye is 1.5 mL whereas that in a human eye is 4.5 mL. In addition,

compared to humans, the rabbit lens is larger and the retina is less vascular, which might influence the distribution and elimination of a drug. The adjustment of drug dosage according to the vitreous and serum volumes in experimental animals used in studies is not usually performed. Data obtained from rabbit studies on systemic effects of drugs are less reliable since the human total serum volume is larger. Given these factors, sheep eyes, in which drug elimination was considered to be similar to that in human eyes, were used in this study. Vertical length of a sheep eye is 30 mm, which is around 23 mm in a human eye[19] . The primary differences between a sheep eye and a human eye include the number and distribution of photoreceptors, the presence of tapetum (Tapetum helps to make the eye visible in the dark in most animals. It is an area in the pigmented layer of the choroids coat of the eye with a metallic brightness), the position of muscle and ciliary body, and the lack of an anterior ciliary artery in major arterial circle of the iris[20].

The sensitivity of the experiment was set at 0.62 ng/mL for ranibizumab. Even though ranibizumab has a molecular weight of 48 kDa, the amount of ranibizumab transferred to the milk and blood of sheep was below the limit of determination. The sensitivity of the experiment was set at 0.9 ng/mL for bevacizumab and though bevacizumab has a molecular weight of 140 kDa, its concentration in the milk and blood of sheep was above the limit of determination even at the end of week 3. In addition, bevacizumab was also detected in the blood of suckling lambs of these sheep.

In the study which try to investigate VEGF levels in the systemic circulation after intravitreal injections of bevacizumab (IVB) or ranibizumab (IVR) in patients with Type 1 retinopathy of prematurity (ROP), Wu *et al*[21] showed that the serum VEGF decreased significantly for 2mo in patients with ROP after IVB treatment significiantly. In contrast, serum VEGF did not change significantly after IVR treatment. The suppression of systemic VEGF in the pediatric population has potential risks[21]. According to Genentech, ranibizumab is a pregnancy category C medication. The risk category of ranibizumab in nursing mothers has not been identified and it remains unknown to what extent it penetrates into breast milk. Bevacizumab is being used off-label in the treatment of most eye diseases. It has been shown that bevacizumab causes embryo-fetal skeletal malformations[22-23]. It is also evident that effects of the intravenous administration of bevacizumab would be greater than those of the intravitreal use of bevacizumab. There is no concrete data in the literature regarding the effects of intravitreal administration of anti-VEGF agents in pregnancy or lactation in humans. There are few case reports on the effects of their use during the first and second trimester in women with unknown pregnancy. In a previous study, 1.25 mg of intravitreal injection of bevacizumab was used in two patients, a 29-year-old female with proliferative diabetic retinopathy and a 25-year-old female with subfoveal choroidal neovascularization secondary to pathologic myopia, and both patients had pregnancy loss[24]. In another study, intravitreal injection of bevacizumab was administered in female patient with subfoveal choroidal neovascularization secondary to pathologic myopia in her 7$^{th}$ gestational week, since long-term results of photodynamic therapy yielded unsatisfactory results, however, the administration of bevacizumab did not result in loss of the fetus. The baby was followed up until age one and no complications were noted[25]. A study by Anastasilakis *et al*[26] reported that a woman, who developed idiopathic choroidal neovascularization in the 8mo of her pregnancy, was administered intravitreal injection of ranibizumab 3mo after delivery, due to deterioration of visual acuity at 2-month post partum follow-up and extension of subretinal hemorrhage to the fovea. Due to the lack of data concerning whether ranibizumab transfers into milk, lactation was stopped[26].

Even though bevacizumab is a larger molecule than ranibizumab, it remains unknown why serum concentration of bevacizumab is high, and through which mechanisms it transfers into serum and the data obtained were no more than hypotheses. There are four pharmokinetic processes to which every drug is subject: absorption, distribution, metabolism and excretion. These four processes affect drug levels in blood and milk. The factors involved in these processes include molecular size of drug, its solubility in fat, ionization, active or passive diffusion and binding to plasma proteins or tissues. It is beyond doubt that these factors played a role in the concentrations of ranibizumab and bevacizumab in blood and milk[27].

Given physical properties of ranibizumab (48 kDa) and bevacizumab (140 kDa), bevacizumab, as a larger molecule, is expected to transfer into blood at lower concentrations after intravitreal injection. Similarly, bevacizumab, given its larger molecular weight, is expected not to cross the placental barrier, however, previous studies have reported contrary findings. It has been demonstrated that serum concentrations of ranibizumab after intravitreal administration were below the limit of determination, whereas, bevacizumab concentration was maintained in blood until day 21[28]. In consistency with these findings, bevacizumab concentrations in both blood and milk after its intravitreal administration were high, whereas, ranibizumab concentrations in blood and milk remained below the limit of determination in this study.

An *in vitro* study demonstrated that the maximum size of molecule capable of freely diffusing across human retina was 76.5 kDa[29], which, however, has been reported to be likely to change due to the presence of active transport mechanisms in *in vivo* conditions.

The internal limiting membrane has porous structure and the size of pores ranges between 10 and 25 nm[30] . The radius

of a full-length antibody such as IgG is 5.5 nm. The external limiting membrane is composed of zonula adherens and the pore size ranges between 3 and 3.6 nm[31-32]. Bevacizumab, which is similar to IgG in structure, can diffuse through the internal limiting membrane, however, it is not expected to be present in the external limiting membrane in the absence of choroidal neovascular membrane (CNVM) or laser effects[33].

Lipophilic compounds such as triamcinolone are eliminated predominantly across the retina whereas hydrophilic compounds diffuse into the aqueous humor first before being eliminated through trabecular meshwork and Schlemm's canal[34-35]. Similarly, bevacizumab, is likely to be eliminated across the iris vascular endothelial and ciliary body non-pigment epithelial tight junctions into the blood circulation and through aqueous humor outflow pathways.

In a study, the right eye of rabbits was injected intravitreally with 1.25 ng/mL bevacizumab, both eyes of the rabbits were enucleated at several time points and bevacizumab concentrations were measured in serum, aqueous humor and vitreous. Bevacizumab concentrations peaked at day 1 in the vitreous and aqueous of the injected eyes and at day 8 in the serum. The study reported that intravitreal bevacizumab passed first into the aqueous humor and then the serum[18].

Higher bevacizumab concentrations in blood can be explained by the assumption that the blood-retinal barrier uses a specific mechanism for transporting and clearing full length antibodies such as IgG as well as drugs similar to IgG in structure[36]. Biologics including monoclonal antibodies, cytokines, growth factors, enzymes, hormones, vaccines, antibody fragments (*e.g.* Fabs) have a powerful clinical effect in most diseases. These molecules have different physicochemical properties compared to small molecules and demonstrate complex pharmacokinetic characteristics that depend on several factors (FcRn, Fcγ receptor, glycosylation, aggregation)[37]. Thus, relevant studies have focused on neonatal FcRn receptor. FcRn receptor plays a central role in the materno-fetal transfer of IgG. It also plays a major role in passive immunization from mother to fetus through the transfer of IgG. FcRn receptor binds to IgG *via* the Fc region. FcRn binds IgG at acidic pH (6.0-6.5) but not at physiologic pH or higher pH[38]. The pH of breast milk is also acidic, being 7.08 in average (6.35-7.65). It appears to be a molecule that can be transported through the placenta in intrauterine period and through breast milk in postnatal period. Bevacizumab is likely to be transferred through the same mechanism.

FcRn prevents degradation by recycling immunoglobulins thus, being the cause of long half-lives of immunoglobulin antibodies in serum. For instance, the half-life of endogenous Ig G is 20d. FcRn has a major role in its long half-life. FcRn protects this protein from lysosomal degradation[39]. The long half-life of bevacizumab in serum can be attributed to the fact that bevacizumab is protected from degradation and recycled by FcRn.

Recent studies have demonstrated that FcRn is also expressed in gastrointestinal tract, breast gland, lungs, liver, vascular endothelium and hematopoietic compartment[40]. These findings are further supported by the detection of bevacizumab concentration in the serum and milk of sheep and in the serum of suckling lambs in this study. FcRn in breast gland may be responsible for the transfer of bevacizumab into the serum of sheep whereas FcRn in the stomach of suckling lambs is responsible for the transfer of bevacizumab from milk into lambs. In this context, it is evident that bevacizumab would pass into the baby's circulation through placental FcRn in pregnant.

Fab fragments are eliminated more rapidly than intact monoclonal antibodies because these molecules lack Fc fragments and protection by FcRn[41].

Other mechanisms have also been proposed. Bevacizumab, with its sugar content, is likely to be transported selectively by galectins and mannose receptor on the surface of RPE cells[42]. Active transport by Müller cells can also be responsible for penetration of bevacizumab through the retina[43].

The gastric pH is high due to alkaline amniotic fluid in the stomach of a newborn (pH 6-8). Gastric pH and acid levels approach adult values within the first 6-8mo of life[44]. Immunogloblulins in breast milk provides passive immunity against most bacteria and viruses in the first 6mo of life in newborns. Accordingly, one of the hypotheses about how intravitreal bevacizumab transfers into the blood of suckling lambs is that, these drugs, which pass into milk and have a protein structure, are not digested due to the inability of the pepsin to become active resulting from high pH of the stomach in newborns.

The limitations of this study are the following: bevacizumab and ranibizumab concentrations, but not VEGF levels, were measured. The study period was limited to 21d since the half-lives of bevacizumab and IgG antibodies are 17-21d. At the end of this period, bevacizumab serum concentrations were still above the limit of determination in some sheep. Similarly, suckling lambs were followed up for 1d and bevacizumab serum concentrations were also above the limit of determination in lambs. For a closer follow up of changes in early hours, blood and milk samples could have been obtained at shorter time intervals. The duration of the study should have been longer, particularly, in the bevacizumab group.

To the best of our knowledge, this is the first study to investigate whether ranibizumab and bevacizumab, which are used commonly in recent years, transfer into the milk and blood of lactating sheep and blood of suckling lambs. Most of the recent data on pharmakokinetic properties of these drugs and the mechanism through which they transfer into serum

are no more than hypotheses. However, it was found in this study that ranibizumab is safer than bevacizumab in lactation. After intravitreal administration, ranibizumab concentrations remained below the limit of determination after 3h when the blood and milk samples were first collected. Ranibizumab was not detected in the blood of lambs. On the other hand, bevacizumab concentrations were high in the blood and milk of sheep and the serum of suckling lambs. Future large-scale animal and human studies and those to be conducted in laboratories enabling measurement of drug concentrations with higher sensitivity are warranted.

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