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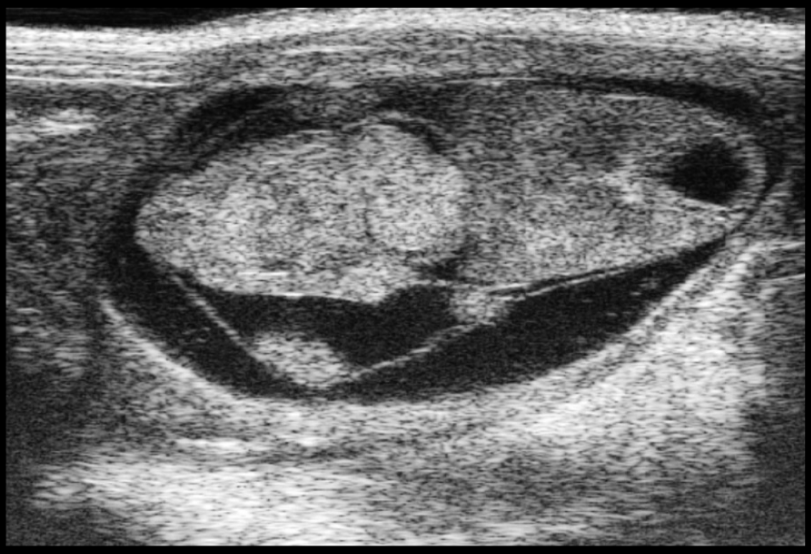

Early detection of embryonic post implantation failures by ultrasound biomicroscopy and the role of the maternal immune system

Inaugural-Dissertation
zur Erlangung des Grades eines
Doktors der Veterinärmedizin
an der
Freien Universität Berlin

vorgelegt von
Luis Eduardo Flores Landaverde
Tierarzt aus Nuevo Laredo, Mexiko

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Contents

List of abbreviations

AA	Aortic arches
Al	Allantois
AC	Amniotic cavity
AM	Amnion
BPD	Biparietal distance
CA	Central artery
CRL	Crown-rump-length
DB	Decidua basalis
DC	Decidua capsularis
DEC	Decidua
EC	Embryonic cavity
ECC	Exocoelomic cavity
EM	Embryo
EPC	Ectoplacental cone
FE	Fetal erythrocytes
HE	Heart
GT	Giant trophoblasts
H&E	Hematoxylin and eosin
LA	Labyrinth
LY	Lymphocytes
ME	Maternal erythrocytes
MES	Mesencephalon
MH	Maternal hemorrhage
MS	Mesometrium
MYO	Myometrium
NC	Neural canal
NE	Neutrophils
NUL	New uterine lumen
OUL	Old uterine lumen
PC	Pericardium
PE	Pericardial effusion
PL	Placenta
RM	Reichert´s membrane
ST	Syncytiotrophoblast
UBM	Ultrasound biomicroscopy
UC	Umbilical connection
UL	Uterine lumen

UV	Umbilical vessel
VV	Vitelline vessel
YS	Yolk sac

1. Introduction and objectives

Embryo resorption is not only a major problem in human reproductive medicine (Macklon et al., 2002; Bulleti et al., 1996), it also represents a major challenge for agricultural animal production (Diskin et al., 2011) and in conservation breeding efforts (reviewed in Andrabi and Maxwell [Andrabi and Maxwell, 2007]). Up to date the underlying mechanisms are still not completely understood and its causes are manifold which include chromosomal anomalies (Simpson and Bombard, 1991), placental insufficiency (Reynolds, 2006) and disturbances in the feto-maternal immune tolerance (Zenclussen, 2005). The maternal immune system during pregnancy requires the generation of a sterile inflammatory response that will be physiologically limited in extent and duration by several immunoregulatory mechanisms (Dekel et al., 2010; Gallino et al., 2016; Weiss et al., 2009). Such mechanisms had to evolve to maintain a tissue homeostasis in a tolerogenic microenvironment (Gallino et al., 2016; Gomez et al., 2010; Mor and Cardenas, 2010). Sub populations of specialized leucocytes such as regulatory T cells (Tregs), uterine NK cells, decidual macrophages, tolerogenic dendritic cells as well as certain cytokines, chemokines, galectins and immune polypeptides play a major role to create and maintain an immune privileged environment (Gallino et al., 2016; Nagamatsu and Schust, 2010; Teles et al., 2013, Pérez et al., 2013; Yoshinaga et al., 2014).

Research on embryo resorption has been conducted mainly by post mortem examinations (Passey et al., 1999; Murphy et al., 2005; Gallino et al., 2016). This approach has several limitations. For instance in studies on early embryo loss, amongst hundreds of implantation sites, only one conceptus was in a state of early resorption whereas all others were either completely normal or completely destroyed (Passey et al., 1999). This fact is a major concern to investigate feto-maternal immune interactions, where the cause and effect are especially difficult to distinguish. Since the establishment of ultrasound techniques in the field of reproductive medicine (Donald et al., 1958), ultrasound technology has advanced greatly and currently ultra-high frequency ultrasound (30-70 MHz) is able to depict structures smaller than 0.1 mm which enables *in vivo* monitoring of prenatal development in small animals. In mouse development, ultra-high frequency ultrasound has been employed to establish growth graphs for the determination of gestational age (Mu et al., 2008), to describe the gross development of the mouse embryo (Zhou et al., 2002; Laissue et al., 2009) and to analyze embryo cardiovascular function (Phoon and Turnbull, 2003; Phoon et al., 2000).

The objectives of this thesis were [1] to identify conceptus undergoing the resorption process at the earliest possible stage during a murine pregnancy in vivo, thus avoiding the onset of a general immune response and [2] to implement an immunohistochemistry evaluation of such early stages. To do so, we had [3] to establish a reliable method of *in vivo* identification by ultra-high frequency ultrasound for conceptus undergoing resorption, which

enabled the comparison of the dynamics between mother, placenta and embryo of implantation sites under resorption and their normally developing littermates.

2. Literature review

2.1 The mouse as a study model

Laboratory animals are frequently used as a model to investigate the causes, diagnosis and treatment of diseases. A rather short gestation that ranges between 18 to 21 days, the low cost of maintenance, a wide arrange of available antibodies, well defined genetic characteristics and the rising number of molecular tools, which include knock-out strains that allow for a straightforward functional analysis of genes involved in the establishment of pregnancy (Farese et al., 1995; Chang et al., 1999), makes the mouse an ideal model to study pregnancy related problems.

2.1.1 Gestation and embryonic development

To better understand the process that takes place after an embryonic death and its subsequent resorption, it is paramount to know the course of the embryonic development and the events that take place during a normal gestation.

The embryonic development will start with the fertilization of the egg (oocyte). Before fertilization can occur, the egg enlarges, divides by meiosis, and matures in its ovarian follicle until it reaches a stage of meiotic division defined as metaphase II. After reaching this stage, the follicle will release the egg into the oviduct (Johnson, 2007). The mature egg, a haploid cell with half the normal number of chromosomes, is enclosed by a protective coat, named the zona pellucida. For fertilization to take place, a haploid sperm cell must bind and penetrate the zona pellucida, fuse with the cell membrane of the egg, enter the cytoplasm and fuse its pro-nucleus with the egg pro-nucleus. Under normal conditions this will take place in the ampulla of the oviduct, a region close to the ovary. The now fertilized egg is knows as a zygote (McGeady et al., 2006). The zygote will then travel to the uterus; this will take three to four days in the mouse. As it travels it will incur in several cell divisions defined as cleavages (figure 1).

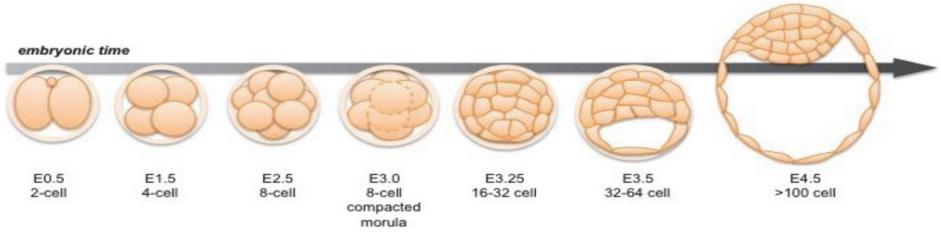

Figure 1. Schematic representation of the different cleavages after fertilization (Schematic by Bergsmedh et al., 2011).

In the mouse, when the zygote reaches an eight-cell stage, the embryo compacts. Around the third day after fertilization the embryo will develop into a compact ball of sixteen to thirty-two cells defined as morula. The morula will keep dividing and its cells will begin to specialize and form a hollow sphere of cells defined as a blastocyst or blastula. The outer layer of the blastocyst is referred as the trophectoderm and the cells inside as the inner cell mass. The cells from the inner cells mass are pluripotent stem cells, which can produce all cell types from the three embryonic germ layers (ectoderm, mesoderm and endoderm). When the blastocyst stage is reached, the embryo hatches from the enclosing zona pellucida and will thereafter implant into the uterus (Rugh, 1990).

The implantation consists of the adhesion between the blastocyst and the uterine endometrium. In the mouse, the implantation occurs approximately at 4.5 days of gestation (Rugh, 1990). It is categorized as eccentric, meaning that it takes place within a fold or recess of the uterine wall (uterine crypt); this always occurs in the antimesometrial side of the endometrium. After implantation, epithelial cells of the uterine crypts start dying, by an apoptotic process induced by trophoblast cells and their proliferation (Parr et al., 1987). Trophoblast cells will continue to proliferate and invade the endometrium. In response to the implantation and invasion of trophoblasts, the endometrium will undergo certain transformations, and a process of decidualization will start. This refers to the differentiation of the underlying fibroblastic stromal cells of the uterus into morphological distinct cells which are then referred as decidual cells (Glasser et al., 1991; Gu et al., 1999).

This process is specific for certain species such as rodents, some primates and to humans. The decidualized endometrium will provide nutritional support for the embryo and work as an immunological barrier which protects the embryo against a maternal immune response. It is also responsible for the production of hormones such as prolactin. It contains a variety of cells that include decidual cells, macrophages and natural killer cells (Croy et al., 2006).

In the mouse the decidual reaction will start antimesometrially in each implantation site and after approximately 48 hours will extend to the lateral and mesometrial areas. At day 6 of gestation the uterine lumen will be closed by the decidual reaction (figure 2). At this stage decidual cells will have completely encapsulated each conceptus. As gestation continues its course, the antimesometrial decidua will thin and stretch out; this portion of the decidua is called decidua capsularis (DC). The decidua located in the mesometrial side is denominated decidua basalis (DB). Starting on day 12 of gestation the decidua will start to disappear; this will start laterally and continue antimesometrially. The DC will disappear completely before the end of the gestation although the mesometrial portion of the decidua will persist throughout the whole pregnancy (Guy et al. 1994).

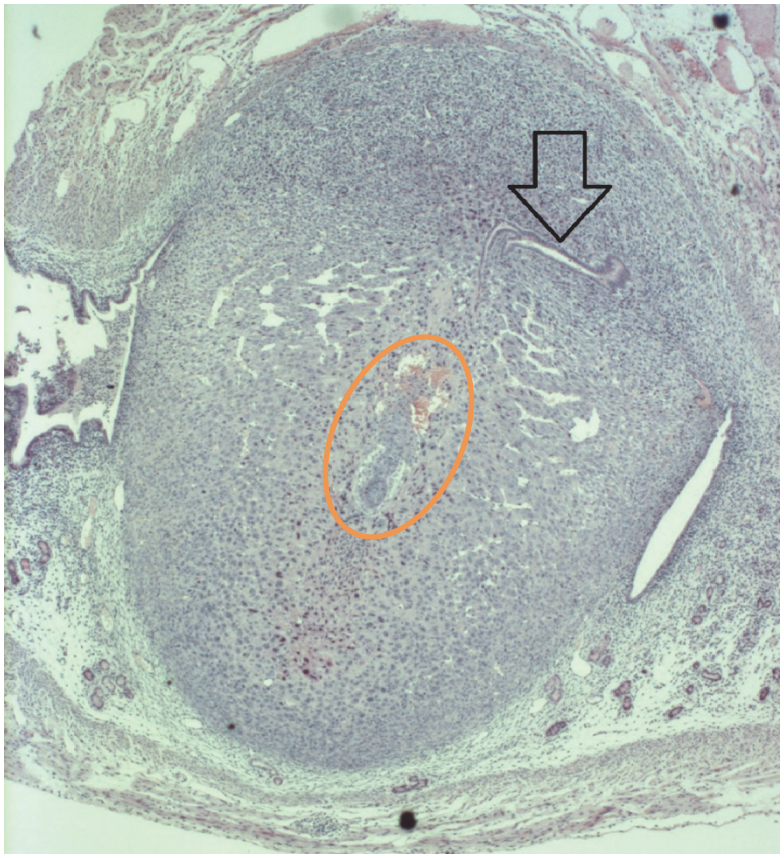

Figure 2. Histological section of a day 6 conceptus portraying how the embryo (oval) is completely enclosed by the decidua and the now old uterine lumen (arrow) has almost disappeared (Photograph by author).

The next phase of development after the implantation has occurred takes place at around day 6 of gestation in the mouse and it is defined with the term of gastrulation. This is a critical phase in which the embryonic ectoderm will differentiate into the three primary germ layers (endoderm, mesoderm and ectoderm). The embryonic ectoderm will give rise to tissues of the central nervous system, and skin. The embryonic mesoderm gives rise to skeletal muscle, heart, blood and other connective tissues. The endoderm will give rise to the epithelium of the digestive tract, respiratory tract, reproductive ducts and glands. Further fetal development descriptions are addressed on the results sections of this thesis.

2.1.2 Placentation

The placenta is an endocrine organ formed by embryonic and maternal tissue, its main function being to allow a physiological exchange between the embryo and the mother. It produces many hormones and helps to minimize the possibility of a rejection of the conceptus by the maternal immune system. Therefore a normal development in the placenta is critical otherwise malformations could lead to embryo lethality and possible subsequent resorption.

At around day 4 of gestation in the mouse, the formation of different trophoblast cell types occurs. Trophectoderm cells will proliferate and give rise to the ectoplacental cone of the early post-implantation conceptus (Copp et al., 1979). As development keeps its course,

the extra-embryonic ectoderm will expand to form the chorionic epithelium. The allantois arises from the mesoderm and will fuse with the chorion at around day 8 of gestation in the mouse (Rossant and Cross, 2001). After this fusion, a ramification of the allantoic vessels will start, and will form a zone called the labyrinth. Between the labyrinth and the decidua, the espongiotrophoblast zone functions as structural support. It is derived from the ectoplacental cone and together with giant cells it is responsible for the production of hormones, angiogenic factors and metalloproteinases. Maternal blood vessels coming from the decidua will traverse the espongiotrophoblast to reach the labyrinth. At around day 9 of gestation in the mouse after the allantois has reached contact with the ectoplacental cone a definitive chorioallantoic placenta will develop (Müntener et al., 1977).

Based on gross shape, the murine placenta is classified as discoid. Histological classification is based on the relationship of the chorion and the uterine wall. Here, murine placenta is hemochorial, meaning it is highly invasive and all maternal layers are lost. This allows a direct contact between the chorionic epithelium (trophoblast) and the maternal blood.

2.1.3 Proliferation and cellular death during murine implantation and placentation

A balance between proliferation and cellular death is essential for implantation and the maintenance of gestation. During the initial adhesion between the blastocyst and the uterine epithelium, the epithelial cells that are near the embryo will experience death by apoptosis (Parr et al., 1987). Such cellular death is induced by trophoblast cells (Schlafke and Enders, 1975). Cells in the uterine epithelium and the decidua will regulate the trophoblast invasion by inducing cellular death. The antimesometrial decidua (DC) which is the first one to form after implantation is also the first one to degenerate and become a thin layer of tissue. Cellular death occurs later in the mesometrial decidua (DB), and it will progressively become thinner throughout gestation (Bell, 1983). This decidual degeneration in both deciduas is a product of an apoptotic process (Gu et al.,1994).

2.2 Ultrasound biomicroscopy in *in vivo* monitoring of prenatal development

Ultra-high frequency ultrasound or so called “Ultrasound Biomicroscopy, (UBM)” can depict structures smaller than 0.1 mm and enables *in vivo* monitoring of prenatal development in small animals. In 1995, Turnbull et al. published the first report on the use of UBM to image mouse embryos in utero (Turnbull et al., 1995). UBM has been used to high-lighten the peculiarities of the long pregnancy of the naked mole rat (Roellig et al., 2011) and to describe embryo development, embryo resorption and corpus luteum regression in the European brown hare (Schroeder et al., 2011, Drews et al., 2012). In mouse development, UBM has been employed to establish growth graphs for determination of gestational age (Mu et al., 2008) and to describe the gross development of the mouse embryo (Zhou et al., 2002, Laissue et al., 2009). Phoon et al. have analyzed embryo cardiovascular function by UBM (Phoon et al., 2000, Phoon et al., 2003). Most recently, UBM has been used to evaluate the effect of defined

quantitative trait loci on embryo lethality in a mouse model of interspecific recombinant congenic strains (Laissue et al., 2009, Vatin et al., 2012). In these studies, the number of dead and living embryos was assessed on defined days of gestation. To date, there was no *in vivo* description of the process of murine embryo resorption.

2.3 The spontaneous embryonic resorption process

2.3.1 Definition

Spontaneous embryonic resorption can be defined as prenatal death followed by the disintegration and subsequent assimilation of the conceptus by the maternal body (Jubb et al., 2007). This process can take place throughout the pregnancy but usually occurs at the embryonic stage. Since there is a complete degeneration and resorption of the conceptus, it can be easily differentiated from other late pregnancy failures such as abortion, mummification, maceration or putrefaction (Réjean, 2015; Givens and Marley, 2008).

2.3.2 Causes and underlying mechanisms

If we think from an evolutionary perspective, spontaneous embryo resorption could be seen as a safety mechanism put into place to ensure the survival of the mother and retrieval of the material resources already invested in the embryo, rather than consider it as a problem or even as a 'disease'. Up to date, the underlying mechanisms are not completely understood. It is conceivable that during the pregnancy the mother may receive information from the conceptus, for instance, if it is damaged or presents abnormalities that are beyond repair. In this scenario an activation of the process might take place. However, it is also possible to argue that the mother might decide to terminate a pregnancy prematurely if she finds herself in difficult circumstances that do not allow her to maintain her own body as well as to produce a healthy offspring (Roff., 1992; Stearns., 1992).

The known causes associated with spontaneous embryo resorption are manifold and can be classified into infections and non-infectious.

2.3.3 Non-infectious causes

There are several non-infectious factors that can be associated with the resorption process, although it is important to remember that an embryonic death might not only result in resorption but in mummification, maceration, or abortion (Givens and Marley, 2008). Until now most studies regarding embryo resorption are conducted via post mortem examinations, so it can be difficult in some cases to assess whether an embryonic death resulted in a resorption process (Schroeder et al., 2013).

2.3.3.1 Chromosomal abnormalities

Chromosomal abnormalities are considered to be a major cause of early pregnancy failure (Rubio et al., 2003). They are classified into two types: numerical and structural (Luthardt et al., 2001). Numerical abnormalities refer to an abnormal number of chromosomes whereas structural abnormalities involve the rearrangement of parts of the chromosomes. In humans, at least 50% of the clinically recognized pregnancy losses which occur during the first trimester are a result of chromosomal abnormalities (Hassold, 1986). Numerical abnormalities associated with early embryonic lethality are the most common (Hassold et al., 1980; Strom et al., 1992; Stephenson et al., 2002). Autosomal monosomies are rarely found in spontaneous embryo resorptions and are better known for causing abortions (Boué and Lazar, 1975; Hassold et al., 1980; Stephenson et al., 2002). These mechanisms may also occur during embryogenesis prior to implantation, which can lead to a progressive loss of abnormal embryos via a resorption mechanism later in development. However the incidence of chromosomal abnormalities associated with embryo lethality and resorption decrease greatly during later pregnancy stages (Machín and Crolla, 1974).

2.3.3.2 Placental insufficiency

The placenta's primary role is to provide and facilitate a physiological exchange between the fetus and the mother (Meschia, 1983; Reynolds and Redmer, 1995). Thus, an adequate development and function of the placenta is fundamental for the health and proper development of the conceptus.

A placental insufficiency is referred as the process that leads to a progressive deterioration of the placental functions which hinders and compromises the transfer of oxygen and nutrients to the fetus. Deterioration of the placental functions will lead to fetal hypoxemia, compromising fetal growth that in some cases could result in death and subsequent resorption of the conceptus (Trudinger et al., 1985; Harrington et al., 1997). These mechanisms are very similar to those seen while exposed to **'**stress**'**, but what causes a placental insufficiency is not necessarily induced by it.

2.3.3.3 'Stress'

'Stress' in the sense of a physiological or psychological challenge increases the allostatic load of the body and is a factor well known to be associated with early pregnancy loss (Arck et al., 2001; Parker and Douglas, 2010). To have an impact on the conceptus, maternal psychological functions need to be translated into physiological effects. There are three mechanisms by which this can happen: An alteration in the maternal behaviors, a reduction in blood flow depriving the conceptus of oxygen and nutrients, and the transport of stress-related neurohormones to the fetus trough the placenta (DiPietro, 2004). Studies in an ICR mice strain demonstrated that inducing stress by physical restraint will not only cause growth retardation in fetuses but also leads to early and late resorptions (Lee et al., 2008).

An increased production of glucocorticoids during maternal stress mediating embryo-fetal toxicities has been suggested as a possible mechanism that leads to fetal resorptions in rodents (Barlow et al., 1975; Barlow et al., 1976; Miller and Chernoff, 1995).

2.3.3.4 High environmental temperature

A high environmental temperature can be considered as a cause of 'stress', usually referred to as heat stress. Studies in dairy cattle have shown that exposure to a high environmental temperature in the cow can be associated with embryonic or fetal losses and resorption (Delasota et al., 1998). It is suggested that the mother, in an attempt to maintain temperature homeostasis, will deprive the uterus of a normal vascular perfusion which results in a reduced nutrient supply to the conceptus (Dziuk et al., 1992). Studies conducted in pregnant Spreague Dawley rats that were exposed to high environmental temperatures resulted in a smaller litter size at parturition than in control groups (Hamid et al., 2012). It was suggested that embryonic survival was compromised more severely during early developmental stages, although the limitations of the study precluded a clear insight whether a resorption process was involved.

2.3.3.5 Malnutrition

Deficit of a specific nutrient or insufficient energy supply will usually entail complications for the upkeep of a healthy gestation. Malnutrition has a negative impact on the quality of the oocyte, the follicular development and it greatly affects the secretory and motility capacity of the oviduct (Butler and Smith, 1989; Foxcroft, 1997). In combination with a severe deficit of vitamins and other nutrients that contribute to the regulation of metabolism, this can lead to early embryo lethality and resorption as shown in females of domestic cattle and domestic horses (Graham et al., 1995). Deprivation of food can by itself induce 'stress' as shown in studies in ICR mice (Lee et al., 2008).

2.3.4 Infectious causes

Infectious agents such as viruses, bacteria and parasites are known to lead to an early embryonic death (Vanroose et al., 2000). This can take place either indirectly as a result of a systemic effect caused by the infection such as high fever or directly by contaminating the oviduct and the uterus and/or affecting the embryo itself. Interestingly, *Listeria monocytogenes*, a bacterial infectious agent which can cause fetal resorptions or spontaneous abortions and stillbirths in humans, does not cause them as a result of a direct placental or fetal invasion, but instead promotes a reduction in maternal Foxp3 positive regulatory T cell suppressive potency, thereby disrupting feto-maternal immune tolerance (Rowe et al., 2012).

Most relevant pathogens are not only responsible for early embryonic deaths, they can have a detrimental effect at various stages of pregnancy. Embryo lethality by pathological agents might result in resorption, abortion, mummification, maceration or putrefaction

(Givens and Marley, 2008). Some of the best known bacterial, parasite and viral agents associated with the resorption process are briefly described in the following three sections.

2.3.4.1 Bacterial pathogens

Bartonella spp. is a gram-negative bacterium which can cause embryo resorptions in BALB/c strain of laboratory house mice (Boulouis et al., 2001).

Brucella canis, a small gram-negative intracellular coccobacillus, is responsible for infertility, early embryonic death and fetal resorptions in female domestic dogs (Johnson and Walker, 1992; Hollett, 2006).

Chlamydophila abortus is responsible for causing enzootic abortion in domestic cows (Menzies et al., 2007). It is well documented that an infection may result in abortion, but if the embryo is less than 95 days old, stillbirth or a resorption process can occur.

Erysipelothrix rhusiopathiae is a gram-positive rod which causes abortion in female domestic pigs. Mummification as well as embryo resorption can take place when infection occurs during pregnancy (Torremorrell, 2007).

Mycoplasma and *Ureaplasma* have been associated with infertility, abortion, stillbirth and fetal resorption in domestic dogs (Johnston et al., 2001).

2.3.4.2 Parasitic pathogens

Neospora caninum, a coccidian parasite, is responsible for early fetal death, mummification and resorption in female domestic dogs (Dubey and Lappin, 2006).

Toxoplasma gondii infections in female domestic sheep are asymptomatic, but an infection before day 40 of pregnancy will lead to embryo resorption (Menzies et al., 2007). In the female goat, infection by *T. gondii* during pregnancy can cause abortion, stillbirth, fetal death or fetal resorption (Mobini, 2007; Dubey et al., 1986). Infections that take place around days 30 to 90 of pregnancy generally result in fetal resorption or mummification, whereas infections that take place in the last half of the pregnancy will result in asymptomatic females aborting around 2 to 3 weeks before parturition.

2.3.4.3 Viral pathogens

Border disease virus is known to cause fetal resorption, abortion, maceration or mummification in infected female domestic sheep before days 60 to 85 of pregnancy (Menzies et al., 2007).

Canine herpesvirus, a virus of the family *Herpesviridae*, can lead to abortion, stillbirth and embryonic resorptions in female domestic dogs (Ronsse et al., 2005).

Canine parvovirus type 1, is known to cause embryo resorption or stillbirth in female domestic dogs (Carmichael et al., 1991).

Feline infectious peritonitis virus, a coronavirus, is associated with abortion, stillbirths and fetal resorption in female domestic cats (Troy and Herron, 1986).

Feline leukemia virus, is a retrovirus known to cause abortion, infertility and fetal resorptions in female domestic cats (Troy and Herron, 1986).

Porcine parvovirus infections in female domestic pigs during days 10 to 30 of pregnancy are known to result in resorption (Mengeling, 2006).

Pseudorabies is caused by an *alpha herpes virus*. Infection during the first trimester of pregnancy in female domestic pigs can result in resorption (Pesak and Truszcynski, 2006; Torremorrell, 2007a).

2.4 Immune tolerance during pregnancy

The embryo is considered to be a semi-allograft since it contains antigens of both paternal and maternal origin. Antigens of paternal origin behave as allo-antigens, and as such, they should be recognised as foreign material by the maternal immune system and therefore be rejected. However, during a normal gestation, this does not occur. The embryo represents a great challenge for the immune system of the mother since it must be tolerated throughout the course of gestation and at the same time the immune system should attack external agents such as bacteria or viruses (Trowsdale and Betz, 2006).

In order to find a suitable compromise between tolerating an embryo and rejecting external agents, there are some mechanisms in place that designate the uterus as an “immune privileged site” during gestation (Arck et al., 2014), in which the embryo will be able to develop normally until parturition. The uterus is pre-disposed to accept a blastocyst. There is a so called “window of implantation” (Chaouat et al., 2002). This is the period of maximal uterine receptivity for the implantation of the blastocyst (Duc-Goiran et al., 1999). Hormones play a major role to help and induce tolerance of the embryo. Progesterone is known to down regulate the cytotoxic activity of the maternal immune system (Piccini and Romagnai, 1996). Prostaglandins in the semen will promote immunosuppresion in the mucosa of the female reproductive tract (Yranzo, 2004).

Peter Medawar is considered a pioneer in studies of the immune tolerance during the pregnancy. His work is the basis of current research approaches to it. In 1953 he proposed for the first time the mechanisms of maternal immune tolerance whilst introducing the concept of the embryo as a semi-allograft (Medawar, 1953). He proposed three mechanisms to explain why the embryo is not rejected by the maternal immune system:

1. The placenta behaves as a barrier. There is a physical separation between the mother and the embryo by an impermeable barrier, and therefore direct contact between fetal antigens and cells from the maternal immune system does not occur.
2. The fetus is antigenically immature, meaning that from an immunological point of view it cannot stimulate the immune cells of the mother.
3. The maternal immune cells are tolerant. The immune system of the mother is tolerant against fetal antigens.

These hypotheses have been an inspiration for many researchers. The current state of knowledge suggests that none of the three mechanisms proposed by Medawar are completely responsible for the observed phenomena. We now know that there is no totally impermeable barrier between the mother and the fetus, trophoblastic cells of fetal origin can circulate in the maternal blood flow, and contact between cells of the immune system and of fetal origin are not restricted to the feto-maternal interface, as cells from fetal origin also migrate to the maternal lymph nodes (Vernochet et al., 2007). This mechanism is important to generate a tolerance in the mother towards antigens of paternal origin (Yranzo, 2004). The feto-maternal relationship can also not simply be explained as tolerance towards a foreign tissue, as it entails a complicated series of interactions between the embryo and the mother mediated mostly by cytokines.

The cellular interaction and mechanisms involved in maternal tolerance to the semi-allograft feto-placental unit are not yet completely understood, but some of the most relevant factors and mechanisms known to be associated with embryo lethality and the subsequent resorption process are described in the following sections.

2.4.1 The relationship between Th1 and Th2 cells

Since the early 1990s the paradigm to explain immune regulation during gestation was focused on the relationship between CD4+T helper 1 (Th1) and T helper 2 (Th2) cells (Wegman et al., 1993). In this model, cytokines produced by Th1 and Th2 lymphocytes have an antagonistic function.

Cytokines from Th1, such as interleukin-2 (IL-2) and interferon-gamma (IFN-γ) and the tumoral necrosis factor alpha (TNF-α) can lead to embryonic death and a subsequent resorption process via activation of a cell mediated specific immune response. These cytokines are responsible for the stimulation of T CD8+ cytotoxic lymphocytes and are found in very low concentrations in the uterus during gestation (Wegman et al., 1993). A response from Th1 is very effective to control intracellular infections and is indispensable to activate a rejection of foreign material. In contrast, cytokines induced by Th2 cells, such as IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13, protect and generate a proper microenvironment for embryo development. These cytokines play a major role in the control of extracellular parasites, stimulate the immune response mediated by antibodies and favour the proliferation and function of polymorphonuclear eosinophil (Wegman et al., 1993, Saito, 2000). In summary, there are cytokines which are beneficial for the development and survival of the embryo, and cytokines that are detrimental and can compromise gestation by causing embryonic death. During gestation, Th2 will inhibit the response of Th1, allowing a normal embryonic development. Regulation of Th1 and Th2 during gestation is governed by hormones. For instance, progesterone is one of the main inducing factors of IL-10, a beneficial cytokine for the upkeep of a normal gestation (Entrican, 2002).

Although the relationship between the cytokines induced by Th1 and Th2 cells is still considered as an important factor for the maintenance of gestation, it would be an oversimplification to limit the effect of the immune system to this relationship only (Margni and Zenclussen, 2001; Chaouat et al., 2002). This is because the relationship between Th1 and Th2 does not include important cells from innate immunity such as the uterine natural killer cells, macrophages, nor the cytokines produced by other cell populations that are not part of the immune system such as the trophoblast, decidual cells and the uterine epithelium. Also, it has been demonstrated in a mouse knock-out model which was genetically deficient in Th2 IL-4, IL-10 cytokines that their absence had no detrimental effect during pregnancy, suggesting that Th2 may be sufficient but not be essential for the maintenance of a normal gestation (Svensson et al., 2001). This also implies that cytokines induced by Th1 cells can be regulated in other ways, for instance by T regulatory cells (Tregs), a special type of T lymphocytes (Zenclussen, 2005).

2.4.2 T regulatory cells

T regulatory cells (Tregs) were initially described as a sub population of specialized T lymphocytes and held responsible for the prevention of autoimmune reactions (Sakaguchi et al., 1995). They have a major role in immunoregulation and are responsible for inhibiting the proliferation and production of cytokines coming from T CD4+ and T CD8+ cells, the production of immunoglobulins from B cells, the cytotoxic activity from natural killer cells and the maturation of dendritic cells, altogether which leads to an induction of a state of tolerance (Sakaguchi, 2006).

Tregs are differentiated and matured in the thymus but can also originate from naïve T cells after being exposed to antigens in the periphery (Apostolou et al., 2002). Tregs are present in the uterus during gestation, a place in which they generate an immune privileged microenvironment responsible for protecting the embryo from rejection by the mother (Zenclussen, 2005; Zenclussen et al., 2006a). The number of Tregs in the uterus will rise after the implantation occurs. After being exposed in the uterus to antigens of paternal origin that are present in constituents of semen in addition to sperm, the Treg population will migrate to the lymph nodes. Afterwards they will again migrate to the feto-maternal interface, where they will produce high levels of protective molecules that will help the embryo not be rejected by the mother (Zencluseen, 2006). Elevated levels of Tregs are seen during most of the gestation and will only start to decrease approximately seven days before parturition (Aluvihare et al., 2004, Zenclussen et al., 2005a, Schumacher et al., 2007).

In a well-established abortion-prone murine model known to present embryonic resorption, T regulatory cells occurred at lower concentrations than during a normal gestation (Zenclussen, 2005). By experimentally transferring T regulatory cells from normal pregnant mice to the abortion-prone model it was possible to prevent the predicted embryonic loss (Zenclussen et al., 2005a).

2.4.3 T helper 17 cells

A subpopulation of T lymphocytes, the T helper 17 (Th17) cells is characterized by the production of a pro-inflammatory cytokine called IL-17. Murine Th17 cells present the transcription factor RORγt which is unique and fundamental for their identification. The main role of Th17 cells is to protect against infections produced by extracellular bacteria (Aujla et al., 2007). Their functions are regulated by T regulatory CD4+ CD25+ cells (Saito et al., 2010). During murine pregnancy, and from day 6.5 to day 10.5 of gestation, Th17 cells are located in the endometrial glands and in the decidua basalis (DB). Around day 12.5 of gestation, immunohistochemistry analysis has proven to be negative for such cells, suggesting that the number of Th17 will reduce drastically after that day (Ostojic et al., 2003). Wang et al. (2010a) demonstrated that the proportion of Th17 in maternal peripheral blood and in the decidua was significantly higher in samples from human patients which had experienced recurrent miscarriages than women with normal pregnancies. Not only were Th17 levels higher in patients with recurrent miscarriage but also the suppressive action of Tregs through the production of IL-17 had diminished (Wang et al., 2010a). These observations suggest that Th17 may have a key role in the immunological rejection of the embryo. Not having a proper balance between Tregs and Th17 will have a detrimental effect on the maintenance of gestation, which might lead to embryonic death and subsequent resorption (Wang et al., 2010b).

2.4.4 Natural killer cells

Natural killer cells (NK) are a specialized type of lymphocyte. In the adult female mouse, a subpopulation of Nk cells, called uterine natural killer cells (uNK), appears in the endometrium at certain phases of the estrous cycle and during early gestation. uNK are the main population of lymphoid cells which can be found in the pregnant uterus and they differ from NK cells in that their function is to help in the establishment and maintenance of a normal gestation. uNK have been extensively studied in the decidua of humans, the laboratory mouse and Sprague Dawley rats (Hunt et al., 2000; Welsh and Enders, 1993; Croy et al., 2009).

During normal gestation of the mouse, an abundant number of uNK can be detected at the implantation site (Peel, 1989). These cells are extremely important during the first half of the gestation since they regulate the initiation of the structural changes of the spiral arteries and aid in the subsequent development of the placenta. Knock-out mouse models helped to elucidate the functions of uNK during murine gestation (Croy et al., 2006). uNK produce cytokines such as IFN-γ (Ashkar and Croy, 1999), IL-18 and IL-27 (Croy et al., 2003). Apart from their main functions they also regulate the trophoblast invasion into the endometrium during implantation in the sense that they prohibit excessive invasion (Hunt et al., 2000). They are also responsible for the removal of aberrant trophoblast cells (Stewart, 1991), and they can synthesize and secrete TFG-β, which inhibits the T lymphocytes that recognize

paternal antigens. In human studies, it has been suggested that circulating NK cells undergo numerical or functional changes in patients that have a history of early pregnancy failures associated to subsequent resorption process and other pregnancy complications (Moffett et al., 2004). However it remains unclear whether these changes are reflected at the maternal-fetal interface (Moffet et al., 2004).

2.4.5 Other mechanisms

The Fas / FasL system is involved in the protection of the conceptus (Makrigiannakis et al., 2001). Fas (CD95) and FasL (Fas ligand) are two proteins that are expressed on cellular membranes. When both molecules are combined they trigger an apoptotic signal towards cells that express Fas. The trophoblast expresses FasL; maternal T lymphocytes have a Fas receptor. The union between the ligand and the receptor will induce an apoptotic process in the T lymphocytes, thereby getting rid of cells that can react against the trophoblast (Bamberger et al., 1997). Other pro apoptotic molecules such as galectin 1 also mediate the immunosuppression of CD8+ T cells (Yranzo, 2004). Dendritic cells are also included in the group of cells that produce important cytokines in the feto-maternal interactions that help to mediate tolerance (Blois et al., 2005).

3. Methods

3.1 Animals

All experimental work on live animals complied with institutional and governmental regulations (Tierschutz-Versuchstierordnung). The institutes committee for animal welfare and ethics and the State Office of Health and Social Affairs Berlin approved the experimental design (letter 03.11.2010) in accordance with §8a of the German law of animal welfare (Tierschutzgesetz). Mice from the inbred C57BL/6 strain were obtained from Harlan Laboratories, Rossdorf, Germany. A total of 30 females and 4 males were kept in open top-wire cages under a 12 h light-dark regime with food and water *ad libitum*. A microchip implant was used for individual identification (Hong Teng Technology, Guangzhou, China). Mice were mated for a period of 3 days in breeding groups comprised of 4 females and one male.

3.2 Examination of pregnancies

Successful mating was confirmed by the presence of a vaginal plug after establishment of breeding groups. In some animals, a vaginal plug could not be detected but pregnancy was confirmed by ultrasound. With the exception of four animals (ID 4, 6, 7 and 13) ultrasound examinations were performed on a daily basis starting on day 4 after establishment of the breeding groups. The duration of the scanning procedures ranged from 10 to 20 minutes per individual. Pregnancy could be confirmed earliest by the ultrasonographic visualization of

decidualized implantation sites on day 5 after establishment of the breeding groups. If implantation sites were detected one or two days later (day 6 and 7 after establishment of breeding groups), we assumed that mating had occurred later, too. Consequently, the day of the first visualization of the implantation sites was always referred to as day 5 of pregnancy. If no pregnancy could be confirmed 8 days after establishment of breeding groups, the animal was considered non-pregnant and was mated again. For the ultrasound examination, an Ultrasound Biomicroscope (Vevo 2100, Visual Sonics, Toronto, Canada) equipped with a 30-70MHz transducer (MS700) was used. The ultrasound settings were standardized as follows: frequency – 50 MHz, power 100%, gain – 30db. Prior examination, mice were anesthetized using inhalation anesthesia via a mask. 5% isoflurane (CP-Pharma, Burgdorf, Germany) was delivered for induction and 1.5%-2% for maintenance with an oxygen flow of 1l/min. To avoid hypothermia, animals were placed on an electric heating mat and the ultrasound gel for the transducer was warmed in a water bath before use. To ensure optimal image quality, the abdominal hair was removed using commercial chemical hair removal gel (Veet, Köln, Germany). In the course of the ultrasound examination the number of conceptuses in each uterine horn was determined. The viability and the staging of the conceptuses was evaluated according to biometric measurements and morphologic parameters. Biometric measurements included the size of the implantation site, the size of the embryonic cavity (EC), the crown-rump-length (CRL) the biparietal distance (BPD) and placental measurements. The size of the implantation site was determined by averaging two perpendicular maximal diameters. The size of the EC was measured in its maximal diameter. Morphologic parameters were the differentiation of decidua basalis (DB) and decidua capsularis (DC), formation of the ectoplacental cone (EPC), the presence of embryonic membranes and the presence and quality of heartbeat. Relevant ultrasound data was recorded for each conceptus. The resorption process was documented by UBM and the respective animals were sacrificed at defined days after the onset of embryo resorption for histological analysis.

3.3 Whole conceptus collection and processing

For the collection of normal conceptuses and conceptuses under resorption, the mouse was euthanized by cervical dislocation and the reproductive tract was removed. The number of healthy embryos and resorption sites was counted and photographed. The uterus was examined by UBM in a water bath with a 0.9% physiological saline solution (Braun, Melsungen, Germany) to verify the *in vivo* ultrasound data. A solution of 4% paraformaldehyde in 1x phosphate buffered saline with a pH 7.4 was used for fixation. A standard protocol for paraffin embedding and sectioning was followed. Sections had a set 3-5 µm thickness.

3.4 Staining protocols

For morphological analysis paraffin sections were dewaxed and stained histochemically with hematoxylin and eosin (H&E) and periodic acid-Schiff (PAS). For immunohistochemistry, the paraffin sections were dewaxed and subjected to a heat-induced epitope retrieval step except for sections for prior incubation with anti-B220 (clone RA3-6B2, BD Bioscience, 1:400). Primary antibodies against cleaved caspase-3 (Asp175, Cell Signaling, USA, 1:400) and MPO (polyclonal rabbit, Dako, code A0398, 1:1000) were used. This was followed by incubation with biotinylated secondary antibodies (Dianova). For detection, alkaline phosphatase-labelled streptavidin and chromogen RED (both Dako) were employed. For detection of macrophages, sections were subjected to protein-induced epitope retrieval employing protease (Sigma) prior to incubation with anti-F4/80 (clone BM8, eBioscience, 1:800). This was followed by incubation with biotinylated rabbit anti-rat (Dako) secondary antibody. Biotin was detected using alkaline phosphatase-labelled streptavidin (Dako). For visualization of alkaline phosphatase, chromogen RED (Dako) was used. Nuclei were counterstained with hematoxylin (Merck). Negative controls were performed by omitting the primary antibody.

4. Results:

In total, we followed 30 pregnancies in 30 different females by longitudinal UBM examinations. The mean number of implantation sites per animal was 7.5 with a range of 1 to 12 implantations per animal. Embryo resorptions were identified between day 7 and 13. In total, 23 resorptions (R1-R23) were detected in 15 pregnancies. Taking all 30 pregnancies into account this resulted in a resorption rate of 10.22% (N=225 normal implantations versus 23 resorptions). To verify and supplement the ultrasound data, an exemplarily collection of resorption sites for histological analysis was obtained. The time points of detection and collection of resorption are summarized in Table 1. In three animals, older resorption sites (R9, R10, R12 and R14) that had occurred earlier in pregnancy were collected together with a fresh resorption site that was identified later in pregnancy (R11, R13 and R15). In total 8 embryo resorptions identified between day 7 and 11, detected in 6 pregnancies were selected for immunohistochemistry analysis (R1, R2, R7, R11, R13, R14, R15, R16).

Table 1. Ultrasonographic detection of embryo resorption and day of collection

Mouse ID	ID of 1st resorption	Detection of 1st resorption (day)	ID of 2nd resorption	Detection of 2nd resorption (day)	ID of 3rd resorption	Detection of 3rd resorption (day)	Number of resorptions per animal	Collection of resorption sites (day)
1	R1	d7	R2	d7			2	8
2	R3	d7					1	8
3	R4	d7					1	8
4	R5	d8	R6	d8			2	9
5	R7	d8					1	9
6	R8	d8					1	9
7	R9	d7	R10	d7	R11	d9	3	9
8	R12	d7	R13	d9			2	9
9	R14	d8	R15	d9			2	10
10	R16	d9	R17	d9			2	11
11	R18	d10					1	10
12	R19	d9	R20	d9			2	10
13	R21	d12					1	12
14	R22	d12					1	12
15	R23	d13					1	13

The number and location of the conceptuses was determined in every ultrasound examination. On day 5, we underestimated the total number of implantation sites by two in one animal, and by one in four animals. On day 6, the number of conceptuses in these animals was corrected and confirmed in subsequent examinations. On day 7, we counted one embryo twice in one animal. The localization of the conceptuses in the right and left uterine horn respectively was always correctly determined with the exception of one embryo on day 6. This mistake was also corrected one day later during the next examination. Apart from these cases, the number and position of embryos during *in vivo* examinations were consistent with the number and position of the conceptuses and resorption sites as derived from the exteriorized uterus and water bath examinations.

The central observations are life stream scans of embryos under resorption compared with their adjacent normal litter mates. Representative scans are documented in the supplementary movies, which are much more informative than the single frames in the figure plates. Figure 3. shows an overview of the results. The major events of normal development are summarized on the abscissa and on the ordinate the major observations in the respective embryos under resorption are shown.

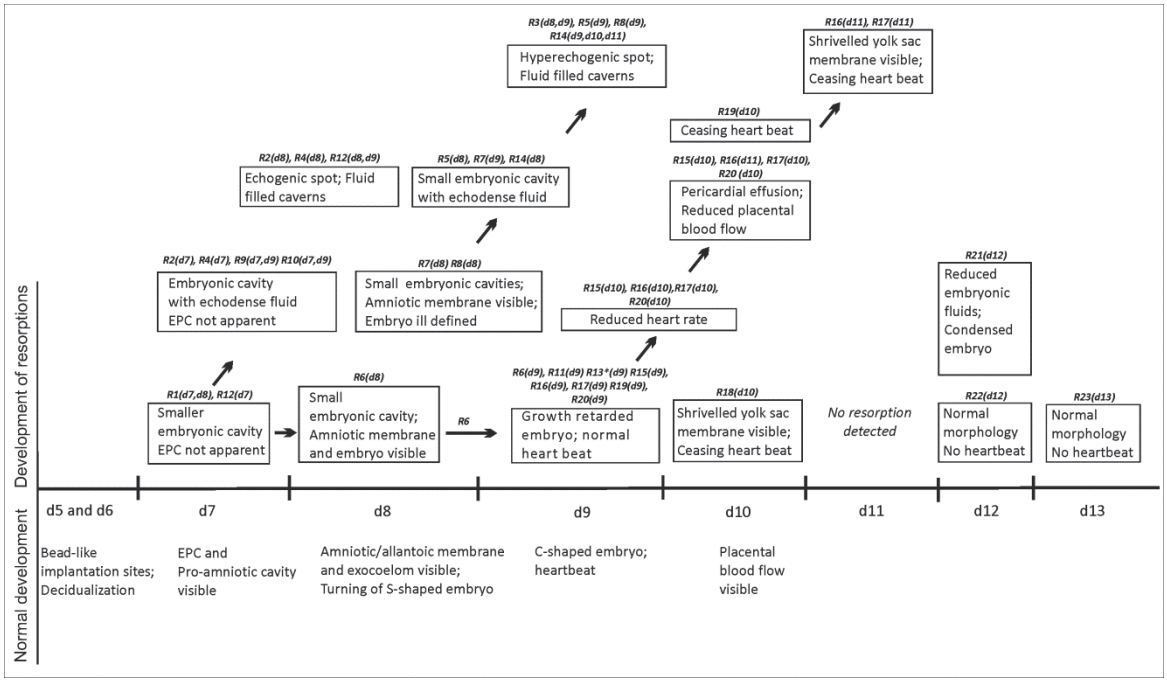

Figure 3. Timeline of embryo resorption.

Ultrasonographic markers of normal development are outlined on the x-axis. The boxes on the y-axis describe the different stages of resorption. The day of detection of the different resorption stages are given in brackets for each resorption site of this study (R1-R23). The day of collection of the resorption sites is indicated by the cross symbol. Observations of follow up exams are aligned by arrows.

EPC – ectoplacental cone; * embryo under resorption located outside the embryonic cavity

4.1 Normal embryo development on days 5 to 8

By UBM, pregnancy could be diagnosed earliest on day 5. The implantation sites appeared as beadlike protrusions of the uterus measuring in average 1.95 mm in diameter (SD=±0.25, N=71), attributed to an extensive decidualization of the endometrium (Figure 2A). An additional movie file shows the normal development on days 6 to 8 (Appendix, see Additional file 1). On **day 6**, the diameter of the implantation site had increased to 2.28 mm (SD = ± 0.39; N=71) (Appendix, Additional file 1) (Figure 4B). The thick decidualized endometrium appeared hyperechogenic compared to the surrounding thin myometrium. Between implantation sites, endometrium and myometrium were difficult to differentiate. The uterine lumen was still visible. At that stage, the embryo was located in its yolk sac cavity. (Figure 4C). On **day 7**, the embryo-maternal interface was characterized by a bright echogenic ring and the wedge-shaped ectoplacental cone protruded in the decidua basalis (appendix, Additional file 1) (Figure 4D). The embryonic cavity measured 0.54 mm in average (SD=± 0.20; N= 80) and could be subdivided into proamniotic, ectoplacental and exocoelom cavity. The embryo proper could not yet be reliably identified (Figure 4D). On **day 8,** the S-shaped embryo started to turn around its own axis. The allantois, which later gives rise to the umbilical vessels, was visible. It transversed the exocoelomic cavity and

connected with the chorion to provide the embryonic vascular component for the chorioallantoic placenta (Appendix, Additional file 1) (Figure 4E).

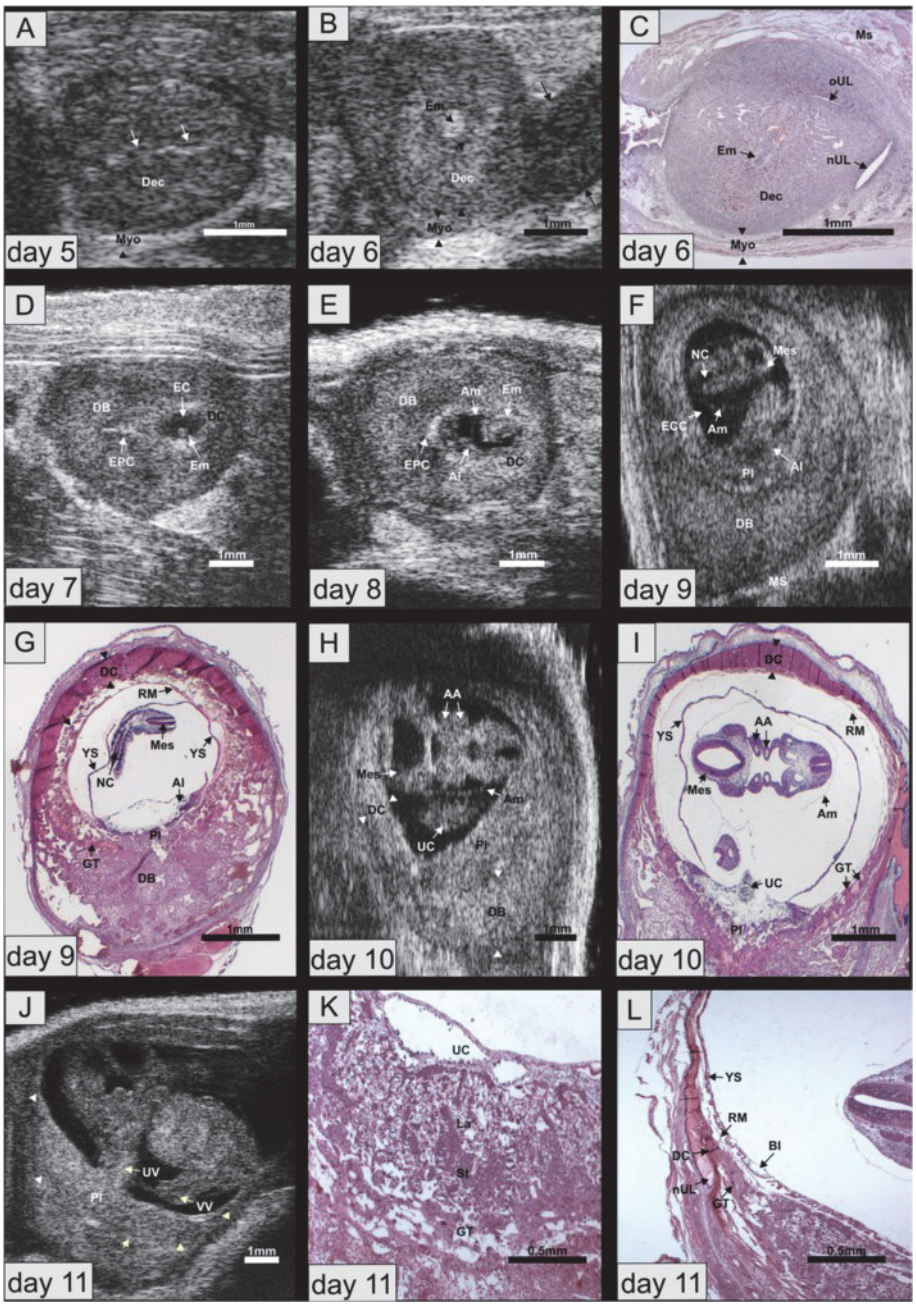

Figure 4. Normal development. (A) day 5. Note the uterine lumen between the two decidualized endometrial layers (arrows). (B) day 6. Higher echogenicity of the endometrium indicates an extensive decidual reaction. The myometrium has a lower echogenicity (arrowheads). Between implantation sites the uterine diameter is very small. The high echodensity spot in the middle of the implantation site indicates the embryo. (C) Histological section of the implantation site shown in (B). (D) Day 7. The embryonic cavity and the embryo proper are visible. Differentiation between the decidua capsularis and basalis is possible. (E) Day 8.The embryo is enclosed on its amnion and the allantois transverses the exocoelom. (F) Day 9. The hyperechogenic decidua capsularis is stretched out at the

antimesometrial side and merges into the decidua basalis at the mesometrial side. The embryo is attached to the placenta via its umbilical cord. The embryonic brain ventricles and the neural canal are visible as hypoechogenic areas. (G) Histological section of the same implantation site shown in (F) pointing out corresponding structures. The Reichert´s membrane and the yolk sac membrane are only visible in the histologic section. In vivo, these membranes are stretched out against the decidua and the placenta. (H) Day 10. The aortic arches and the mesencephalon are depicted. (I) Histology of the same embryo as in (H) showing corresponding structures. Again, the Reichert's membrane and yolk sac membrane are only visible in the histologic section. (J) Day 11. The placenta has increased in size and displays hyperechogenic calcification deposits (arrowheads) at the fetomaternal boundary. The vitelline and umbilical vessels are seen. (K) Histological section of placenta of same embryo as shown in (J). The giant trophoblast is in the process of disappearing. (L) Histology of same embryo as in (J and K) outlining the transition zone between decidua capsularis, decidua basalis and new uterine lumen.
AA - Aortic arches; Al – Allantois; Am - Amnion; DB - Decidua basalis; DC - Decidua capsularis; Dec – Decidua; EC – Embryonic cavity; ECC – Excocoelomic cavity; Em-Embryo; EPC – Ectoplacental cone; FE – Fetal erythrocytes; He – Heart; La – Labyrinth; Mes – Mesencephalon; Ms – Mesometrium; Myo – Myometrium; NC – Neural canal; nUL – new uterine lumen; oUL – old uterine lumen; RM – Reichert´s membrane; Pc – Pericardium; Pl – Placenta; St – Syncytiotrophoblast; UC – Umbilical connection; UL – Uterine lumen; UV – Umbilical vessel; VV – Vitelline vessel; YS – Yolk sac.

4.2 Embryo resorptions on day 7 and 8

Identification of embryo resorption was first possible on day 7 when the ectoplacental cone was visible. On day 7, seven implantation sites were suspicious for resorption (R1, R3, R2, R4, R9, R10, R12) because their embryonic cavities were smaller than in their litter mates (EC=0.30 mm; SD=± 0.07; N=7) and the ectoplacental cone was not well defined. In R1 and R12 the fluid in the embryonic cavity, which could not be further differentiated, was filled with clear fluid (Figure 5A) while on day 8, the fluid was echodense (Figure 5B). In the histological section, the embryonic cavity was filled with proteinaceous material and the placentation site consisted of condensed trophoblast tissue and maternal haemorrhage (Figure 5C). In four resorptions (R2, R4, R9, R10) the embryonic fluid was already echodense on day 7. By day 8, the embryonic cavities had disappeared and the implantation sites were transformed into echodense tissue surrounded by fluid filled caverns. A hyerechogenic spot was detected in the periphery of the resorption sites. Histological analysis showed that the caverns corresponded to maternal haemorrhage in the decidua basalis and the hyperechogenic spot to fibrinoid tissue.

Two resorption sites (**R1**, **R2**) were suspicious for resorption on day 7 by high frequency ultrasound. The resorption sites were collected one day later. Histological analysis

showed that the embryo proper and its membranes had already disappeared and placental tissue was not present. Massive maternal hemorrhage occurred between the embryonic giant trophoblast cells and in the spongious part of the decidua basalis accompanied by a moderate infiltration of neutrophils. Maternal blood was also found in the uterine lumen. In one resorption site (**R1**), a circular area composed of amorphous material heavily infiltrated by neutrophils was present. This area was located between the giant trophoblast ring and the decidua capsularis. It is likely that the amorphous material represents the remnants of the embryo. At the mesometrial side, an agglomerate of Caspase-3 positive decidual cells was prominent.

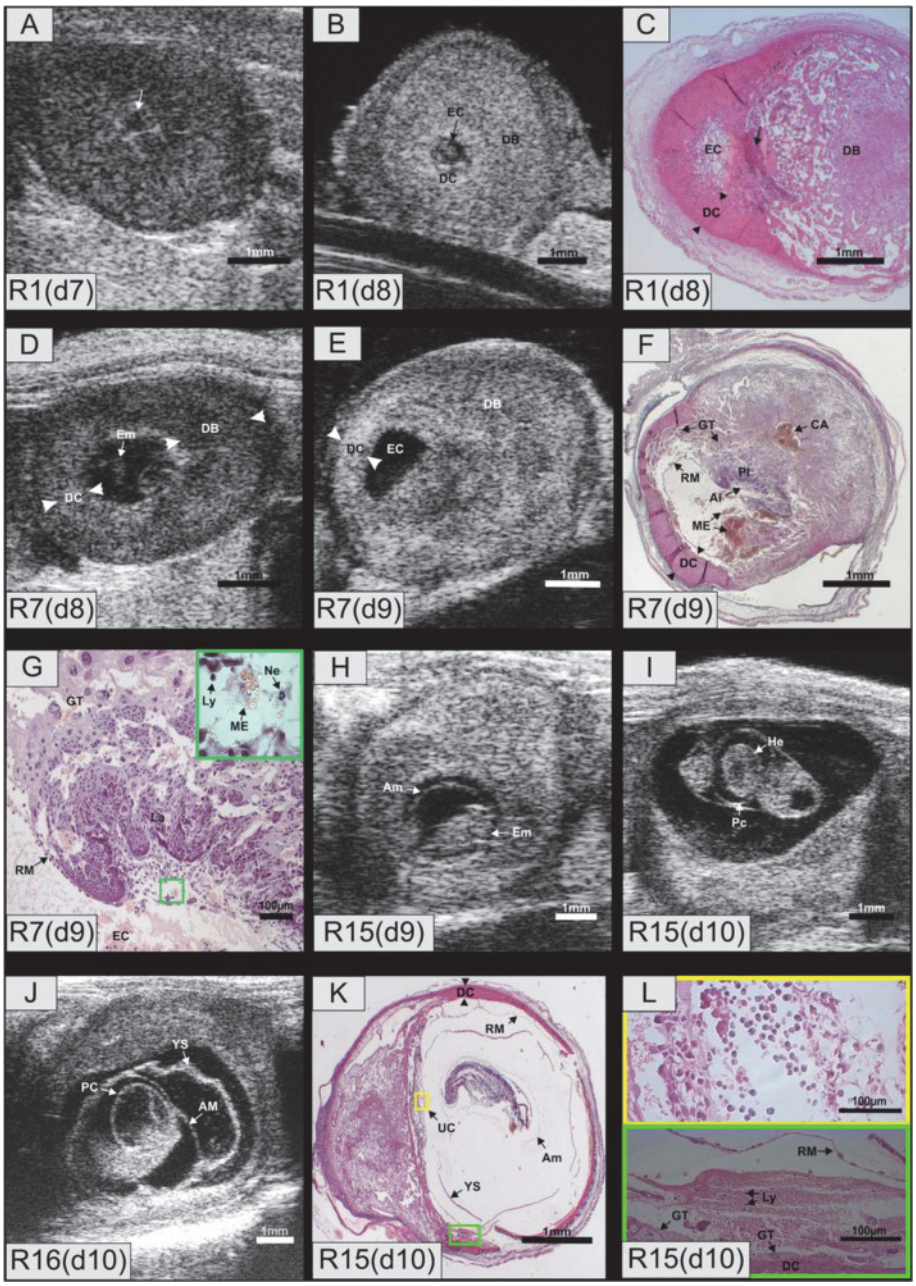

Figure 5. Embryo resorptions R1, R4, R7, R15 and R16. (A) R1, day 7. The resorption site is characterized by a small embryonic cavity (arrow) lacking a well defined ectoplacental

cone. (B) R1, day 8. The fluid in the embryonic cavity has increased in echodensity. (C) R1, day 8. There is massive maternal hemorrhage in the giant trophoblast ring and spongious trophoblast in the transition zone. The former placental site is composed of fibrinous tissue heavily infiltrated with maternal granulocytes (Arrow). (D) R7, day 8. The embryo has formed, but its morphology is not well defined. (E) R7, day 9, scanned post mortem in the water bath. The embryo is not visible any more. The decidua capsularis is evident by its high echogenicity but the placental borders are not clearly demarcated. (F) R7, day 9. The embryo and its membranes except for the Reichert's membrane have disappeared. The former embryonic cavity is filled with denaturated proteins. There is a massive maternal hemorrhage between the giant trophoblast layer and the Reichert´s membrane. The central artery is filled with blood. (G) Higher magnification of the placenta of R7. A compact labyrinth layer can be seen. Erythrocytes from embryonic origin were absent in the allantois. A magnification of the area outlined shows the presence of lymphocytes, neutrophils and erythrocytes in the allantois. (H) Growth retarded embryo R15, day 9. (I) R15, day 10. The embryo continued to develop but exhibited a reduced heart rate. Pericardial effusion is evident. (J) R16, day 11. The heartbeat has ceased. The pericardium, amnion and yolk sac can be differentiated. (K) R15, day 10. All embryonic membranes are visible, but the yolk sac is devoid of blood islands. The placental morphology is normal. (L) R15, day 10. Magnification of the areas outlined in (K). The umbilical connection is filled with fetal erythrocyte (yellow rectangle). In the transition zone of decidua capsularis and decidua basalis, maternal blood with a high concentration of immune cells is found between the Reichert´s membrane and giant trophoblast (green rectangle).
Al – Allantois; AC – Amniotic cavity; CA – Central artery; DB - Decidua basalis; DC - Decidua capsularis; EC- Embryonic cavity; Em- Embryo; GT – Giant trophoblasts; La – Labyrinth; Ly – Lymphocytes; ME - Maternal erythrocytes; Ne – Neutrophils; Pl – Placenta; RM – Reichert´s membrane; UC – Umbilical connection; YS – Yolk sac.

On day 8, four resorptions (R5, R6, R7 and R14) were detected on the basis of their small cavities (EC=0.70 mm; SD=± 0.34; N=4) compared to 1.35 mm (SD=± 0.35; N= 86) in the normal developing conceptuses.

In one resorption (R6) the embryo proper was visible but the embryonic cavities were smaller compared to the normal siblings. This embryo was clearly growth retarded one day later (CRL= 0.91 mm) compared to a mean CRL of 1.72 mm (SD=± 0.13; N=6) in its litter mates. In another resorption (R7) the embryonic cavity was also smaller and the shape of the embryo was ill defined (Figure 5D). In that resorption (R7), only the embryonic cavity was left in the follow up exam one day later (Figure 5E). Histological analysis confirmed that the embryo proper and its inner membranes had completely disappeared. Only the Reichert's membrane was found in the original embryonic cavity (Figure 5F). Between the Reichert’s

membrane and the giant trophoblast layer in the transition zone of the decidua capsularis, a massive influx of maternal blood was apparent. The central artery was also filled with blood. Maternal erythrocytes, neutrophils and lymphocytes could be seen in the allantois, but no embryonic erythrocytes were detected (Figure 5G).

In the resorptions R5, R7 and R14 no embryo or embryonic membranes were visible by ultrasound. The fluid in the embryonic cavity was echodense. In the histologic section (R10) only the Reichert's membrane was left, as observed in R7. This resorption stage was transformed into the typical echodense tissue with surrounding caverns within 24h (R5, R8 and R14).

In the resorption (**R7**) that was detected on day 8 and collected on day 9, the original structure of the embryo, which must have been smaller than in **R15**, was not apparent anymore. However, Reichert's membrane, placenta and giant trophoblast ring were clearly visible. Maternal blood clots with leucocytes were located in the uterine lumen, together with fibrinous precipitation and material that resembled the embryonic membranes. The degrading decidual cells were vacuolated and had lost their cell boundaries. Between the giant trophoblast ring and the placenta maternal hemorrhage had occurred. Maternal blood with a large amount of leucocytes was also present within the former embryonic cavity, inside the Reichert's membrane. It seems that here the resorption process with necropsis of the embryo had started within the implantation site and then remnants of the embryo had been expelled to the uterine lumen via the deteriorated, porous decidua capsularis.

Resorption **R14**, which was growth retarded on day 8 as seen by high-frequency ultrasound but was only collected on day 10, the same resorption process had taken place but was already more advanced. Here, placental tissue was not present, but the decidua capsularis showed the same, localized spongious appearance as observed in **R14**. Again, maternal blood with granulocytes was detected between the giant trophoblast, in the former embryonic cavity and in the uterine lumen. Additionally, amorphous, fibrinous material was present in the uterine lumen.

4.3 Normal development on days 9 to 13

On day 9, the originally concave embryo had assumed a convex curvature and was enclosed in its amnion. Attributed to the inversion of germ layers, the exocoelomic cavity is the main extraembryonic cavity and the yolk sac cavity consists merely of a slim slit between exocoelom and Reichert's membrane. The yolk sac membrane was therefore not visible by UBM. Details of the embryonic morphology such as the mesencephalon and the neural tube became evident (Figure 4F) and the embryonic heartbeat could be detected (Appendix, Additional file 2). In corresponding histological images the exocoelomic cavity had collapsed and the space between the folded visceral yolk sac membrane and the Reichert's membrane was artificially enlarged (Figure 4G). In the yolk sac membrane, numerous blood islands had developed. Nucleated erythrocytes were evident in the allantois. The originally

antimesometrial decidua, the decidua capsularis, consisted of densely packed cells and thinned towards the mesmometrial pole, where it blended into the richly vascularised mesmometrial decidua, the decidua basalis. Between the Reichert's membrane and the decidua capsularis at the abembryonic pole, a layer of giant trophoblast formed a ring and marked the border between the placenta and the decidua basalis at the mesometrial side. Between days **10 and 13**, the embryo considerably enlarged in size. The pericard and the heart with its atria and ventricles could be clearly distinguished by ultrasound, as well as the aortic arches (Figure 4 H and I). The placenta exhibited a similar echogenicity as the decidua basalis but could be differentiated by its pulsating blood vessels and by a layer of higher echogenicity between embryonic trophoblast and maternal decidua (Figure 4J, Additional file 3). In the histological sections, the placenta had differentiated in its placental labyrinth, spongiotrophoblast and giant cell layer (Figure 4K). A transition zone between decidua capsularis, decidua basalis and new uterine lumen developed (Figure 4L). At this stage, the decidua capsularis and giant trophoblast cells were in the process of disappearing.

4.4 Embryo resorptions on days 9 to 13

On day 9, seven resorptions (R11, R13, R15, R16, R17, R19, R20) were first identified on the basis of growth retardation. R6, that had already exhibited a smaller embryonic cavity on day 8 also showed growth retardation on day 9. The crown rump length of the embryos (mean CRL day 9 growth retarded embryos= 1.39 mm; N=8) was comparable to a developmental stage that was reached one day earlier in the normal siblings (mean CRL day 8 normal= 0.99 mm; SD=± 0.30; N= 77) (Appendix, Additional file 2) (Figure 3H). Placental size was also smaller in the embryos under resorption (Table 2).

Table 2. Crown rump-length (CRL) and placental size of normal embryos and embryo resorptions

	day 9	day 10	day 11	day 12	day 13
Placental width (normal embryo)	1.66 mm; SD= ± 0.32; N= 57	3.14 mm; SD= ± 0.50; N= 38	4.71 mm; SD= ± 0.63; N= 21	5.83 mm; SD= ± 0.14; N= 13	8.03 mm; SD= ± 1.36; N= 2
Placental height (normal embryo)	0.54 mm; SD= ± 0.09; N= 57	0.83 mm; SD= ± 0.15; N= 38	1.17 mm; SD= ± 0.20; N= 21	1.37 mm; SD= ± 0.14; N= 13	2.11 mm; SD= ± 0.07; N= 2
Placental width (resorption)	1.22 mm; SD= ± 0.38; N= 12	2.40 mm; SD= ± 0.58; N= 8	3.78 mm; SD= ± 0.77; N= 4	4.81 mm; SD= ± 0.98; N= 3	4.22 mm; N= 1
Placental height (resorption)	0.42 mm; SD= ± 0.09; N= 12	0.60 mm; SD= ± 0.10; N= 8	1.04 mm; SD= ± 0.21; N= 4	0.84 mm; SD= ± 0.21; N= 3	1.18 mm; N=1
CRL (normal embryo)	2.11 mm; SD= ± 0.46; N= 72	4.01 mm; SD= ± 0.54; N= 50	5.71 mm; SD= ± 0.85; N= 29	7.38 mm; SD= ± 0.81; N= 18	9.31 mm; SD= ± 0.70; N= 5
CRL (resorption)	1.39 mm; SD= ± 0.43; N= 8	2.41 mm; SD= ± 0.75; N= 6	1.47 mm; SD= ± 0.44; N= 2	6.54 mm; SD= ± 1.48; N= 2	5.60 mm; N=1

Within 24 hours, the growth retarded embryos exhibited bradycardia (92 bpm in resorption prone embryos versus 130 bpm in normal siblings) and pericardial effusion

(Appendix, additional file 3) (Figure 5I). The size of the heart corresponded to that of a normally developing embryo and was therefore proportionally bigger to embryonic body size. Interestingly the resorption prone embryos were still able to develop further, albeit at a reduced rate (Appendix, additional files 3-5) (Figure 5 H and I). Due to the loss of embryonic fluids, the formerly expanded exocoelomic cavity deflated and the folded yolk sac membrane became visible by UBM (Appendix, additional file 4) (Figure 5J). One resorption prone embryo (R15) that was first detected on day 9 (Figure 5H) showed a reduced heart beat on day 10 (Figure 5I). It was collected on day 10 when it was still alive but its heartbeat was barely detectable. In the corresponding histological sections all membranes were identified (Figure 5K) though the yolk sac membrane was lacking the typical blood islands. In the transition zone, maternal blood had accumulated between the Reichert's membrane and the decidua capsularis, which was still delineated by giant trophoblast cells (Figure 5L). The umbilical vessel was filled with embryonic erythrocytes (Figure 5L), which underlined the previous ultrasonographic observation of a faint heartbeat (Appendix, additional file 5). Surprisingly, the cells of the embryo proper showed signs of necrosis to a great extent.
In the next phase of the resorption process, the heartbeat finally ceased. Histological analysis showed that the resorption process continued with the necrosis of the embryo proper, which was still surrounded by its membranes. In two cases (R13, R16), the embryo was located in the uterine lumen due to a rupture of the decidua capsularis (Figure 6A and B, Additional file 4). In the histological section of R13, yolk sac and Reichert´s membrane were found inside the embryonic cavity. The exteriorized embryo was surrounded by its amniotic membrane. The morphology of its placenta was unsuspicious (Figure 6C).

In a resorption site detected on day 9 and collected on day 10 (**R15**), the embryo was still enclosed in its membranes and located within the implantation chamber. The placenta was well developed and the allantoic bud was evident. Within the embryo proper, localized caspase positive spots were evident. Caspase positive cells included areas in the somites and in the neural tube. In the control sibling, caspase positive cells were also found in the neural tube but to a much lesser extent. Strikingly, the embryonic erythrocytes showed a unique morphology with an eccentric nucleus and formation of vacuoles. These cells were found in the embryo proper and in the allantoic bud. Some of these erythrocytes stained positive for MPO7. Maternal blood with a high number of leucocytes was found within the amniotic cavity, the labyrinth placenta and the allantoic bud. Maternal erythrocytes were also present in the embryo proper. At the transition zone between decidua capsularis and decidua basalis, a massive maternal hemorrhage with leucocyte infiltration was detected.

A more advanced stage of development is represented in resorption **R11**. It was visibly growth retarded as seen by high-frequency ultrasound on day 9 and collected on the same day. In **R11** the embryo was in the process of being exteriorized into the uterine lumen via a rupture site of the decidua capsularis. Close to the rupture site, the decidua capsularis had the typical spongy appearance with deteriorating decidual cells and infiltration of maternal

neutrophils. The epithelium of the uterine lumen was eroded. F4/80 positive cells were detected under the epithelium of the uterine lumen. The part of the embryo that was still in the implantation chamber was enveloped by its amnion, yolk sac membrane and Reichert's membrane. Within the uterine lumen, only the yolk sac membrane was present. Maternal blood had invaded the amniotic cavity. Maternal erythrocytes were found in the allantoic bud. Some of the embryonic erythrocytes in the allantoic bud had an eccentric nucleus and cytoplasmic vacuoles as seen in **R15**. Small cell clusters in the embryonic somites were Caspase-3 positive. In the exteriorized part of the embryo, single MPO7 positive cells were detected.

In resorption **R13** (detected on day 9 and collected on day 9), the embryo was completely exteriorized. Notably, on the day of collection, the heartbeat of the embryo was still detected. The embryo was partly enclosed by its amnion; Reichert's membrane and yolk sac were still inside the implantation chamber. The morphology of the rupture site of the decidua capsularis corresponded to the rupture sites described for **R11** with spongious decidual cells, infiltration of granulocytes and eroded maternal epithelium. Some of the decidual cells and cells underneath the maternal epithelium at the rupture site were F4/80 positive. The rupture site was further characterized by large blood clots of maternal origin. The allantoic bud contained embryonic erythrocytes but maternal erythrocytes were also detected. Mixed blood was also found between Reichert's membrane and yolk sac membrane. The cells of the embryo proper had to great parts lost their integrity. Maternal erythrocytes were present in the neural tube. Caspase-3 staining was difficult to evaluate due to strong background staining. However, only a few, localized cell spots in the somites and the neural tube seemed to be truly caspase-3 positive.

Resorption site **R16** was first evident by high frequency ultrasound on day 9 but only collected on day 11. Here, the resorption process was more advanced than in **R13**. The decidua capsularis was not only ruptured but had almost completely vanished, leaving the implantation chamber wide open towards the uterine lumen. Where the decidua capsularis was still present, the adjacent giant trophoblast layer was infiltrated with maternal blood containing a large amount of neutrophils and also B-lymphocytes that were positive for B220. The embryo was not present anymore; only Reichert's membrane was left in the uterine lumen.

In my study, there were no indications for a first detection of the resorption process on day 11.

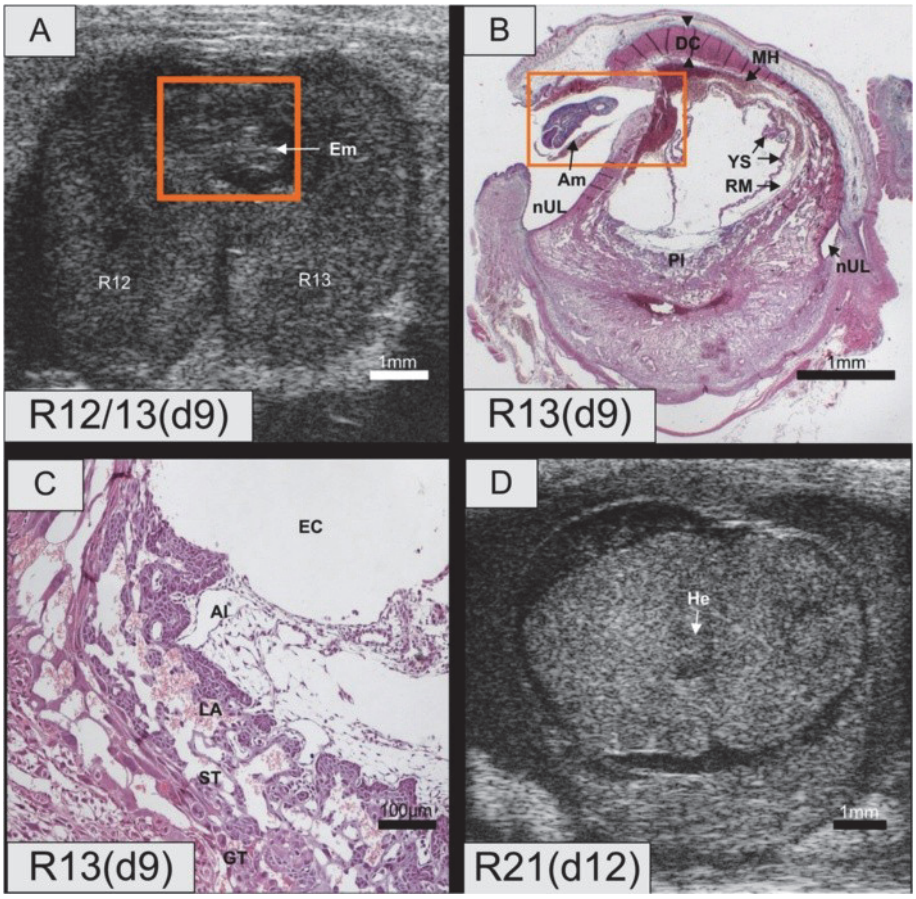

Figure 6. Embryo resorptions R12, R13 and R21.
(A) R12 and R13, day 9. In R12, the embryo is no longer visible. In R13, the embryo is still present but it is located in the uterine lumen. (B) R13, day 9. The decidua capsularis is ruptured. The embryo is encased in its amnion but outside the yolk sac membrane and Reichert's membrane, which are still located in the original embryonic cavity. The yolk sac membrane exhibits blood islands and is folded due to fluid loss. Between Reichert´s membrane and yolk sac membrane, there is maternal hemorrhage. (C) The placental barrier is intact. Blood spaces filled with maternal blood are evident as well as embryonic blood vessels containing nucleated erythrocyte. (D) R21, day 12. There was no visible heartbeat and less fluid in the embryonic cavity.
Al – Allantois; Am – Amnion; DC – Decidua capsularis; EC – Embryonic cavity; Em – Embryo; GT – Giant trophoblasts; MH - Maternal hemorrhage; nUL – New uterine lumen; PE – Pericardial effusion; Pl – Placenta; RM – Reichert´s membrane; UC – Umbilical connection; YS – Yolk sac.

On day 12, two embryos that were unsuspicious on the previous day were found dead (R21, R22). One embryo (R21) showed reduced embryonic fluids and condensed embryonic tissue (Figure 6D). On day 13 an embryo that presented a normal morphology had no visible heart beat (R23).

In conclusion, the process of embryo resorption is characterized by four distinct phases: in the first phase, growth retardation is observed which manifests in the reduced size of the embryonic cavities and the delayed developmental stage of the embryo itself. In the second phase, the embryo exhibits a reduced, sometimes irregular heartbeat, reduced placental blood flow, detachment of the yolk sac membrane from the outer Reichert's membrane and pericardial effusion which subsequently results in stalling of the heartbeat. In the third phase, first the embryo disintegrates, then its inner membranes disappear and finally the placental integrity is lost. In the final stage of resorption, the implantation site is characterized by hypoechogenic caverns and a central, hyperechogenic spot which correspond to maternal haemorrhage and fibrinoid tissue, respectively. The time course of the outlined resorption stages and the respective morphological characteristics vary according to gestational age as outlined above.

5. Discussion:

In my study I described the *in vivo* process of murine embryo resorption using UBM and correlated my ultrasound data to histology. The normal development served as a reference for the successful *in vivo* detection of embryo resorption. The process of embryonic perishment and subsequent degradation of the conceptuses undergoing the resorption process was also described using the aid of immunohistochemistry.

The resorption followed a specific pattern, independent from the time in gestation when the resorption process started. The first sign to presage resorption prone embryos was delayed development or growth retardation. On the basis of my study growth retardation can be strongly associated with the resorption process, even though a considerable variability in developmental stage within one litter has been demonstrated in the post mortem study of Thiel et al. (thiel et al., 1993). The developmental difference observed in the study of Thiel et al. varied by almost one day. However, since these observations rely on post mortem findings, the subsequent development of the smaller embryos was not documented. We assume that some of embryos which exhibited the least development were in fact prone for resorption. Measurements of CRL on day 9 from my study support this possibility since the gestational age difference between embryos of the same litter was not greater than half a day if we excluded the resorption prone embryos.

In early pregnancy stages, growth retardation manifested in smaller embryonic cavities and an undifferentiated shape of the embryo itself. In my study, a smaller embryonic cavity suggestive for resorption could be first observed on day 7.

On day 8, resorption prone conceptuses could be identified by their smaller size. In some cases, the embryo itself first developed but its morphology was ill defined and it then disappeared within 24 hours. The detection of embryos under resorption that early in gestation is therefore highly dependent on the level of experience of the sonographer, considering that the morphological changes are delicate and occur at a fast rate. It seems that with advancing

development, the time period between the first signs of growth retardation and death of the embryo is elongated, making it more likely to identify living but resorption prone embryos. Beginning on day 9, when the heart beat was reliably detectable, the second stage of the resorption process could be visualized. Here, the dysfunction of the embryonic circulation manifested in bradycardia, reduced placental perfusion, and pericardial edema. Another finding, the visualization of the shrivelled yolk sac membrane by UBM which is not evident in normal embryos might account for the fact that the limited and finally ceased production of embryonic fluids reduces the physiological turgor of the exocoelomic cavity. This eventually leads to an artificial increase of the yolk sac cavity.

The third phase of the resorption process implies the death of the embryo. In other studies, the death of the embryo is defined by a ceasing heartbeat. The heart of the mouse starts to beat between day 8 and 9 (Srinivasan et al., 1998; Ji et al., 2003). Diagnosis of embryonic death on the basis of heart action is therefore not possible prior this day. Theoretically, the determination of the exact time point of embryonic death based on the absence of a heartbeat would require permanent live ultrasound scanning. In my study, bradycardia, arrhythmia and pericardial edema in growth retarded conceptuses preceded the final cessation of heart function. After observation of these markers, ultrasound examinations can be performed twice daily to delineate the time window of death. However, one has to consider that ceasing of the heartbeat might in fact not be the appropriate marker for embryonic death. We showed that one embryo under resorption, which still exhibited a faint heartbeat, already showed severe necrosis of the cells of the embryo proper. This finding shows that the border between the end of life of an individual is equally fluent and difficult to define as its beginning. In early gestation, it is even harder to narrow down the period of death since the embryo proper only begins to develop and its heart is not yet beating. Death in that developmental stage occurs at the cellular level only and is reflected in arrested development of the conceptus, accompanied by an increased echogenicity of embryonic fluids as seen by high frequency ultrasound.

In the fourth stage the conceptus dissolves and is subject to haemorrhage and necrosis. The final resorption stage consists of fibrinoid, condensed scar tissue which persists for an extended period of time. In post mortem studies, this is the stage where the resorption site is identified macroscopically in the exteriorized uterus (Murphy et al., 2005; Kusakabe, 2008; Clark et al., 2004). By ultrasound, this final stage of resorption observed in early pregnancy was characterised by a high echodensity spot. We documented similar high-echodensity spots along the ectoplacental cone and in the placenta at the embryo-maternal border in normal conceptuses. In a study of Akirav et al, these high density foci have been identified as calcium deposits (Akirav et al., 2005). The concretions most likely originate from dystrophic calcification processes in dysfunctional cells where the active calcium transport is impaired. They can therefore be considered as a marker for the last stages of apoptosis and necrosis.

This process seems to originate from the embryo itself, since we always observed first the death of the embryo, then its dissolution, and then the disappearance of its inner membranes. These observations have also been made in an ultrasound study on embryo resorption in the European brown hare (Schroeder et al., 2013). In human reproductive medicine, an anembryonic gestational sac is considered as an ultrasonographic marker for embryonic demise (Odeh et al., 2010). High levels of alpha-fetoprotein of yolk sac origin in the maternal circulation are indicative for an early death of the embryo which was resorbed before it became ultrasonographically detectable (Jauniaux et al., 1995).

In my study, the Reichert's membrane which is unique to rodents and acts as a filter between embryonic and maternal tissue (Salamat et al., 1993), was the last membrane to disappear. Together with the finding that the placenta was morphologically unsuspicious these observations support the hypothesis that death is triggered within the embryo itself. This is consistent with my observations of caspace positive cells in the embryo proper while undergoing resorption. The control siblings also presented caspace positive cell but to a lesser extent. By means of cell competition, embryonic cells can compare their fitness with that of neighbouring cells resulting in apoptosis of the less fitter cells (De la Cova et al., 2004). This mechanism has been demonstrated to play a crucial role in the selection of mouse embryonic epiblast (Claveria et al., 2013). If that cell competition becomes unbalanced, it could result in embryonic death. There seems to be a higher selection pressure on the long lived epiblast cells than on the short lived cells of the extraembryonic membranes (Claveria et al., 2013), reflecting my finding that the membranes undergo resorption only after the embryo has already disappeared.

Early in gestation (day 8), an inflammatory process in the decidua capsularis was observed, accompanied by a local swelling and disintegration of the decidua capsularis. At this stage we did not find any rupture of the decidua capsularis in the conceptus being resorbed. During later stages of the gestation (day 9 up to 11), when the embryo proper had increased in size, the inflammatory process resulted in the rupture of the decidua capsularis and the release of the embryo under resorption into the uterine lumen. To my knowledge the rupture of the decidua capsularis as part of a mechanism of the mother to cope with the amount of apoptotic embryonic tissue has not been described previously. Degradation of the placenta may possibly follow a different pathway but as my study was designed to detect the earliest possible stages of resorption, it was not possible to follow the demise of the placenta any further.

In knockout mice, the depletion of specific genes may result in a characteristic embryonic phenotype (Laissue et al., 2009; Vatin et al., 2012) which can be further examined with additional methods such as hybridization and immunohistochemistry. Longitudinal UBM examinations will enable to determine the exact stage at which certain genes need to be expressed to ensure healthy embryo development and survival.

The monitoring of embryo development by UBM will also be useful in the field of epigenetics, where certain environmental factors acting on the adult individual influence the gene expression and intra-uterine development of their offspring (Simmons, 2009; Jammes et al., 2012). The use of UBM to detect embryo resorptions will significantly reduce the number of experimental animals in studies investigating embryo failure for two reasons: first, pregnant animals can be reliably detected from day 5 onward and non-pregnant animals can be saved. Second, embryo resorptions can be identified *in vivo* at an early stage enabling the sacrifice of the experimental animal at the appropriate time.

6. Conclusions:

In my study I have shown that UBM is a useful tool to detect resorption prone embryos and to follow their involution process over time. With this method, resorption prone embryos can be specifically targeted and harvested before the onset of decomposition. This is particularly important for the study of embryo-maternal immune reactions, where the specific maternal immune response towards the dying embryo must be differentiated from a general immune reaction necessary to clear the uterus from apoptotic tissue. Furthermore, the morphology of the placenta and extraembryonic membranes could be evaluated *in vivo* over a period of time before its integrity is compromised by dissection.

My results provide a temporal time of embryo resorption and also suggest that the early resorption process seemed to be controlled and mediated by apoptotic processes within the embryo rather than necrosis. When embryonic death occurs during advanced stages of gestation after the embryo proper has already reached a considerable size, the mother is confronted with a large amount of apoptotic embryonic tissue and placental tissue, both of which need to be cleared from the implantation side. In this case, the process of embryo depletion and placental degradation obviously takes more time and can be more readily observed. It is possible that the implantation site is cleared by expulsion of embryonic material or the whole embryo via the uterine lumen. The degradation of the placenta may possibly follow a different pathway.

Summary

Spontaneous embryonic resorption is considered a major problem in human medicine, agricultural animal production and in conservation breeding programs. Its causes and the underlying mechanisms have been investigated in the well characterized mouse model. However, most studies rely on post mortem examinations and have severe limitations because of the rapid disintegration of embryonic structures. The aim of this thesis was to establish a reliable method to identify embryo resorptions in alive animals at the earliest possible stage by ultra-high frequency ultrasound which will allow us to better understand and further investigate its underlying mechanisms because it avoids the onset of a general immune response.

In my study, I provide a temporal time course of embryo resorption. Four stages of the resorption process were described in detail: first the conceptus exhibited growth retardation, second, bradycardia and pericardial edema were observed, third, further development ceased and the embryo died, and finally the embryo remnants were resorbed by maternal immune cells. During early gestation (day 7 and 8 of gestation), the first stage was characterized by a small embryonic cavity. The embryo and its membranes were ill defined or did not develop at all. The echodensity of the embryonic fluid increased and within one to two days, the embryo and its cavity disappeared and were transformed into echodense tissue surrounded by fluid filled caverns. During later gestational stages (day 9 to 11), the resorption prone conceptus was one day behind normal siblings in its development. Growth retarded conceptuses exhibited bradycardia and ultimately cessation of heart beat. The corresponding histological sections showed apoptotic cells in the embryo proper while the placenta was still intact. In the subsequent process first the embryo and then its membranes disappeared.

I also describe in depth, the process of embryonic perishment and the subsequent degradation of the implantation site. Specific markers for macrophages (F4/80), neutrophils (MPo-7), and lymphocytes (B220) were used and apoptotic cells were detected via Caspase 3 staining. The process commenced with the apoptosis of the embryo proper. Early in the gestation (day 8), I observed an inflammatory process located in the decidua capsularis, which led to its disintegration. A massive maternal hemorrhage occurred at the decidua basalis. Degraded material and maternal erythrocytes were found in the uterine lumen. At later gestation (day 9) when the embryo proper has increased in size, the inflammatory process seen on the decidua capsularis resulted in its rupture and the release of the embryo proper into uterine lumen. I suggest that the release of the dead embryo into the uterine cavity occurring between days 9 and 11 of pregnancy might help to escape the immune privileged environment of the implantation site. The degradation of the placenta seems to follow a different pathway.

Zusammenfassung

Früherkennung embryonaler Postimplantationsstörungen mittels Ultraschallbiomikroskopie und die Rolle des maternalen Immunsystems

Die spontane Resorption von Embryonen stellt ein massives Problem in der Humanmedizin, der landwirtschaftlichen Tierproduktion und in Erhaltungszuchtprogrammen dar. Im Mausmodell wurden die dafür zugrundeliegenden Ursachen und Mechanismen bereits untersucht. Die meisten dieser Studien basieren allerdings auf postmortalen Untersuchungen, wodurch sie massiven Beschränkungen unterliegen, da die embryonalen Strukturen einem schnellen Zerfall unterliegen. Ziel der vorliegenden Arbeit war es eine zuverlässige Methode zur Identifikation von Embryonenresorptionen bei lebenden Tieren mittels hochauflösendem Ultraschall in möglichst frühen Stadien zu entwickeln. Diese Methode soll unser Verständnis der zugrundeliegenden Mechanismen erweitern und weitere Untersuchungen hierzu ermöglichen insbesondere da eine Aktivierung der allgemeinen Immunantwort vermieden wird.

In meiner Studie präsentiere ich einen zeitlichen Zeitverlauf der Embryo Resorption. Vier Stadien des Resorptionsprozesses wurden im Detail beschrieben. Im ersten Stadium zeigt der Embryo eine Wachstumsverzögerung. Im zweiten Stadium werden Bradykardie und periphere Ödeme beobachtet. Im dritten Stadium kommt es zum Stillstand der weiteren Entwicklung und der Embryo stirbt. Im vierten und finalen Stadium findet die Resorption der Embryonenreste durch die maternalen Immunzellen statt. Während der frühen Trächtigkeit (Tag 7 und 8 der Trächtigkeit) wird das erste Stadium durch eine kleine embryonale Höhle charakterisiert. Der Embryo und seine Fruchthüllen stellen sich im Ultraschall nur unklar dar oder haben sich in der Gänze nicht entwickelt. Die Echodichte der Embryonalflüssigkeit ist erhöht und der Embryo und sein Hohlraum verschwinden innerhalb von ein bis zwei Tagen und werden umgewandelt in ein echodichtes Gewebe welches von einer flüssigkeitsgefüllten Kaverne umgeben ist. Im späteren Trächtigkeitsstadium (Tag 9 bis 11) zeigt der für die Resorption anfällige Embryo eine im Vergleich mit seinen Wurfgeschwistern um einen Tag verzögerte Entwicklung. Der im Wachstum zurückgebliebene Embryo wies eine Bradykardie auf und stellt schließlich den Herzschlag ein. Die korrespondierenden histologischen Gewebeschnitte zeigen zum Embryo zugehörige apoptotische Zellen, während die Plazenta weiterhin intakt ist. Im nachfolgenden Prozess verschwanden zunächst der Embryo und später die zugehörigen Fruchthüllen.

Ich beschreibe im Detail den Prozess des Embryonaltods und die anschließende Zersetzung des Implantationsortes. Spezifische Marker wurden verwendet um Makrophagen (F4/80), neutrophile Granulozyten (MPo-7) und Lymphozyten (B220) zu detektieren. Apoptotische Zellen wurden mittels Caspase 3-Nachweis identifiziert. Der Prozess begann mit dem Nachweis von Apoptose in den embryonalen Zellen. In der frühen Phase der Trächtigkeit (Tag 8) wird eine entzündlicher Prozess in der *Decidua capsularis*, welcher ihren

Zerfall auslöste beobachtet. Eine massive maternale Blutung wurde an der *Decidua basalis* beobachtet. Das untergehende Embryonalgewebe und die maternalen Erythrozyten wurden im Uteruslumen nachgewiesen. In der späteren Trächtigkeit (Tag 9), in der der Embryo vergrößert war, resultierte der entzündliche Prozess auf der *Decidua capsularis*, in dessen Ruptur und in der Freisetzung des Embryos in das Uteruslumen. Ich schließe aus die Beobachtungen, dass die Freisetzung des toten Embryos in das Uteruslumen, die zwischen dem 9. und 11. Trächtigkeitstag auftritt, dabei helfen könnte der bevorzugten Immunantwort in der Umgebung des Implantationsortes zu entgehen. Der Abbau der Plazenta scheint einem anderen Weg zu folgen.

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Appendix

Supplementary material

Movie file 1: Normal development day 6 – 8
On day 6, the implantation site is visible as a bulge of the uterus. The endometrial layers are seen as a hyperechogenic, white line. On day 7, the ectoplacental cone and the proamniotic cavity become evident. On day 8, the embryo is encased in its amnion and the allantois traverses the exocoelom. The ectoplacental cone invades the mesometrial decidua basalis.
Download video: http://www.rbej.com/content/12/1/38/suppl/S1

Movie file 2: R16 and R17 day 9
In the normal embryo, head and rump can be differentiated and the heartbeat is evident. The umbilical cord attaches to the placenta. The resorption prone embryos R16 and R17 display the same morphological features but are visibly smaller compared to the normal embryo.
Download video: http://www.rbej.com/content/12/1/38/suppl/S2

Movie file 3: R16 and R17 day 10
The normal embryo displays a pulsating heart with atria and ventricles enclosed in the pericardium. The blood flow from the umbilical cord to the placenta is visible. The embryo-maternal interface is characterized by calcifications between the trophoblast and the placenta. The resorption prone embryo R16 is visibly smaller than its normal sibling. However, its heart rate is not yet reduced. The resorption prone embryo R17 shows growth retardation, pericardial effusion and a reduced heartbeat.
Download video: http://www.rbej.com/content/12/1/38/suppl/S3

Movie file 4: R16 and R17 day 11, first and second scan
The normal embryo has increased in size. In contrast, the R16 is now clearly in the process of resorption: the heart is compressed by pericardial effusion and a heartbeat is barely detectable. The yolk sac is visible as a shrivelled membrane after having lost its close connection to the Reichert's membrane and underlying maternal mucosa. R17 has died. The amniotic cavity is small and the yolk sac has also lost its balloon like shape, resulting in a shriveled yolk sac membrane. In the second scan, 3 hours later, the morphology of the normal embryo is not altered. In R16, a heartbeat cannot be detected. The embryo of R17 has completely lost its original morphology and its tissue looks condensed. The embryo seems to be outside its original cavity in the uterine lumen. During collection of the resorption site, the embryo was lost. Only the Reichert's membrane was found in the uterine lumen.
Download video: http://www.rbej.com/content/12/1/38/suppl/S4

Movie file 5: R15 day 9 and 10

On day 9 the resorption prone embryo R15 shows visible growth retardation. Compared to its normal sibling, the morphology is poorly defined. On day 10, the resorption prone embryo has increased in size and its heart can be differentiated. The pericard is filled with excess fluid and its heart rate is greatly reduced as illustrated by color doppler flow.

Download video: http://www.rbej.com/content/12/1/38/suppl/S5

Publication list

1.- Luis E Flores, Thomas B Hildebrandt**,** Anja A Kühl**,** Barbara Drews**.** (2014). "Early detection and staging of spontaneous embryo resorption by ultrasound biomicroscopy in murine pregnancy." Reproductive Biology and Endocrinology 12(1): 38.

2.- L Flores-Landaverde, A Kühl, B Dews. Rupture of the decidua capsularis and release of embryos under resorption in the uterine lumen: a necessary step to escape the immune privileged implantation site?. 48th Annual Conference Physiology and Pathology of Reproduction and simultaneously 40th Joint Congress of Veterinary and Human Medicine (February Conference 2015), Zurich, Switzerland, 11th - 13th February 2015. Reprod Domest Anim. 2015 Feb;50 Suppl 1:11

Part of this dissertation has been presented orally or in poster format at international scientific conferences:

1. Flores-Landaverde L, Hildebrandt TB, Kühl AA, Drews B. Rupture of the decidua capsularis and release of embryos under resorption in the uterine lumen: a necessary step to escape the immune privileged implantation site?" CTR Annual Trophoblast Meeting 11th – 12th July 2016. Cambridge, United Kingdom. (Poster presentation)

2. L Flores-Landaverde, A Kühl, B Drews. "Rupture of the decidua capsularis and release of embryos under resorption in the uterine lumen: a necessary step to escape the immune privileged implantation site?". 48th Annual Conference Physiology and Pathology of Reproduction and simultaneously 40th Joint Congress of Veterinary and Human Medicine (February Conference 2015), Zurich, Switzerland, 11th - 13th February 2015. (Oral presentation)

3. Flores-Landaverde L, Hildebrandt TB, Kühl AA, Drews B. "Early detection of embryonic post implantation resorption by ultrasound biomicroscopy and histological correlation in murine pregnancy." CTR Annual Trophoblast Meeting 8th – 9th July 2013. Cambridge, United Kingdom. (Oral presentation)

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Selbständigkeitserklärung

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe.

Berlin, den 18.09.2017

Luis Eduardo Flores Landaverde

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