Type I interferon response in bovine endometrial cells co-cultured with peripheral blood mononuclear cells

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Abstract

Embryonic loss is an issue that livestock producers are faced with worldwide. This particular research assesses one aspect of the role that immune cells play in embryonic survival, more specifically, the interaction between peripheral blood mononuclear cells and bovine endometrial cells, and their connection to interferon-stimulated genes. Peripheral blood mononuclear cells were taken from three different cows and were cultured with bovine endometrial cells. Some of the cultures contained interferon, and some did not. After incubation, results showed that the immune cells did not increase interferon-stimulated genes, and are stimulating a pathway in the response to pregnancy that is currently unknown.

Background

As the population of the world steadily increases, animal scientists are faced with the challenge of producing more animal protein to feed the world. One estimate states that by the year 2050, the production of food will have to increase by seventy percent (Alexandratos & Bruinsima, 2012). One means of addressing this issue focuses on increasing the reproductive efficiency of cattle in order to increase the amount of beef and dairy products available for consumption. Increasing fertilization rates is typically not the problem with cattle, however, they do suffer from a high rate of embryonic loss (McMillan, 1998). According to Diskin & Morris (2008), fertilization rates generally fall between ninety and one hundred percent for most cattle, with the exception of high-producing dairy cows who are typically lower. Currently, research is being performed that is focused on addressing the issue of embryonic loss. Fair (2015) stated that the embryonic and fetal mortality rate in cattle is around thirty-five percent. If this relatively high rate of embryonic loss can be decreased, this not only would save livestock producers a large sum of money, but would also enable them to utilize current reproductive technologies in a way that makes a more efficient impact to the food supply. One such reproductive technology that is widely used is embryo transfer. Embryo transfer is used to select desired genetics to produce valuable males and females (Ideta et al., 2010). This is a very useful reproductive technology, however, if embryonic loss continues to occur at the rate that it does, technologies such as embryo transfer are not reaching their full potential.

Many scientists are devoting their research to addressing the issue of embryonic loss from an immunological perspective. The maternal immune system has been found to be an influential part of pregnancy from the time of insemination and throughout the entire pregnancy. Though the maternal immune system is not the only factor influencing embryonic and fetal survival, it does play a large role in making sure a healthy pregnancy occurs. This type of research is still continuing around the world to gain a better

understanding of maternal immunity in hopes of addressing embryonic loss and infertility in not only cattle, but also other species of animals as well as humans.

The Embryo

In both cattle and sheep, the embryo enters the uterus around days four to five following fertilization and becomes a blastocyst around day six or seven (Fair, 2015; Takahashi et al., 2016). In cattle, implantation of the embryo to the uterus does not begin to occur until day nineteen, therefore leaving nineteen days for the embryo to face the first effects of immune response and survive off of uterine secretions. These uterine secretions are regulated by P4 (progesterone) and are a crucial part of maintaining growth of the conceptus (Takahashi et al., 2016). It is believed that the events that occur during the first few days after mating have a large effect on whether the embryo will be lost or will become a viable fetus. Fair (2015) stated an estimate that "70-80% of the total embryonic loss occurs between days 8 and 16 after insemination." Another study also found that embryonic loss in cattle generally happens before day sixteen after breeding, and in high producing dairy cows, there has been a large occurrence of embryonic loss before day eight (Diskin & Morris, 2008). At this point of development, the conceptus is faced with having to inhibit the secretion of PGF2α from the uterus, which is a possible explanation for embryonic loss if the conceptus fails to do this (Thatcher et al., 2001). Another possibility, however, has to do with the immune system. From the time immediately after insemination to the time of embryo implantation, the maternal immune system plays a significant role in the establishment of pregnancy by altering its function to protect the embryo and promote a successful pregnancy. Because of this, researchers are looking into the possibility that the maternal immune system is affecting the current rate of embryonic loss that producers are experiencing.

During the course of reproduction, the immune system of the female is exposed to many foreign antigens including the semen itself as well as the conceptus (Seamark et al., 1992). Typically, foreign antigenic material is attacked by immune cells so the body can rid itself of the foreign material. Pregnancy, therefore, is an "immunological contradiction" because the foreign material that is introduced into the female reproductive tract is not rejected but rather forms a close relationship with the uterus (Walker et al., 2010). It is clear that the immune systems of humans and animals have special adaptations when it comes to pregnancy so that the foreign embryo can bypass immune system attack and avoid being rejected from the mother's body. This includes suppressing cytotoxic adaptive immunity, increasing regulatory adaptive immunity, and maintaining innate immunity. If this type of immune system alteration were not in place, successful pregnancy would not occur. In every stage of the reproductive cycle, immune cells are present (Seamark et al., 1992). When a pregnancy is detected, it becomes a matter of balancing both pro-inflammatory and anti-inflammatory molecules in order to maintain an optimal environment for the developing embryo (Walker et al., 2010).

In women and cattle, it has been found through research that transferring autologous immune cells to the uterus increases pregnancy rates (Chase et al., 2015). Peripheral blood mononuclear cells (PBMCs) have the capability to secrete proteases, which likely plays a role in uterine regulation (Yoshioka et al, 2006). Through the research of Ideta et

al. (2010), an increased pregnancy rate was found for cows administered PBMCs into the uterus prior to pregnancy compared to those who were not administered them. Additionally, Yoshioka et al., (2006) performed an experiment where PBMCs were taken from women and cultured with human chorionic gonadotropin and administered into the uterus along with fresh PBMCs three days before embryo transfer was performed. They found that this was a successful way to increase implantation and overall pregnancy rates likely due to the activation of the PBMCs when exposed to stromal tissue (Yoshioka et al., 2006). Collectively, these results illustrated that the peripheral blood mononuclear cells initiated a response in the endometrium that conditioned it for pregnancy which leads into the next topic, immunological priming (Ideta et al., 2010).

Immunological Priming

Now aware of the changes that occur to the maternal immune system, researchers are looking into what actually causes this change in immune function. This leads to the topic of immunological priming. Immunological priming is essentially when immune cells first come in contact with a foreign antigen, promoting the immune cells to differentiate into effector B or T cells. These effector cells can be in the form of cytotoxic T cells, cytokines or antibodies and are produced depending on the task that the immune system faces. It is believed that certain components of semen, including the foreign antigens that it contains, play a role in priming the immune cells of the mother in order to facilitate a successful pregnancy (Robertson, 2005). Recent studies on livestock, rodents and humans have shown that the mere exposure of the female to seminal fluid during coitus induces certain changes in the female that promotes conception and successful pregnancy. In mice it has been found that as soon as semen deposition occurs, inflammatory cells immediately travel to the site to initiate a post-mating inflammatory response (Robertson, 2005). In another study, it was reported that neutrophils were released into the uterine lumen within an hour after mating, followed by other types of immune cells (Schjenken & Robertson, 2014). The reason why this inflammatory response occurs so quickly after mating is clearly understood. The central role of this inflammatory response is to clear excess sperm from the uterus and try to regain the sterility of the reproductive tract after it was exposed to foreign material containing potentially harmful microorganisms (Schjenken & Robertson, 2014). Following this, immune changes continue to occur to prepare the uterus for pregnancy. When seminal fluid contacts epithelial cells in the female reproductive tract, certain molecules in the fluid bind to receptors to initiate changes in gene expression (Robertson, 2005). This upregulation of certain genes is thought to be a key component of embryonic immune tolerance (Walker et al., 2010). Tissue remodeling of the uterus to increase embryonic receptivity as well as activation of the maternal immune response due to seminal antigen exposure occur as a result of this inflammatory response that is initiated by seminal fluid (Robertson, 2005).

For years, it has been general knowledge that the sperm component of semen is important for reproduction, but more recently it has been found that the portion of the semen that does not contain sperm, seminal plasma, also plays an important role in the reproductive process (Robertson, 2005). Seminal plasma contains cytokines, sex hormones and prostaglandins that are all crucial in the reproductive process (Schjenken & Robertson, 2014). This claim is supported by a study that analyzes reproduction in mice. It was

found that the success of embryo transfer was directly correlated with if the recipient was mated with a vasectomized male (Robertson, 2005). By mating with the vasectomized rat, the female was still being exposed to the seminal plasma portion of the ejaculate, which showed to help decrease the occurrence of embryonic loss and fetal abnormalities (Robertson, 2005). Bromfield et al. (2004) also uncovered supporting evidence when recipient females were mated with males who lacked a functional seminal vesicle and fetuses with growth rate abnormalities as well as placental abnormalities were found. The research performed by Bellinge et al., (1986) showed that this is also the same case with humans when an increase in live birth rates was observed in women who had sexual intercourse near the time that they underwent in vitro fertilization. In addition, Seamark et al. (1992) also found that a component of semen enhances fertility in the pig after intrauterine insemination. In all of these cases, it was found that the seminal plasma component of seminal fluid stimulated the tissues of the female reproductive tract to promote the establishment of a successful pregnancy.

Immune Cells Involved in Seminal Priming

As described above, when the maternal immune system is exposed to the antigens and other factors that are in seminal fluid, a certain activation of the immune system occurs. Seminal activation essentially generates an immune tolerance to the foreign antigens to avoid the lymphocytes that respond to cause cytotoxic damage to the embryo or the sperm (Robertson et al., 2009). According to Schjenken and Robertson (2014), the foreign conceptus expresses a variety of antigens, one type "encoded by major histocompatibility genes derived from paternal chromosomes". Because the female immune system has no recognition of these antigens, an immune activation response initiates. This activation process begins when endometrial stromal tissue is exposed to the foreign antigens present in seminal fluid, and macrophages and dendritic cells are brought to the site of exposure (Robertson, 2005). In short, macrophages and dendritic cells are both leukocytes that function as antigen presenting cells, and in this case, they present foreign seminal antigens to helper T cells in the uterine draining lymph nodes to continue the activation of subsequent immune responses (Robertson, 2005). Despite different species having different sites of semen deposition, evidence of seminal fluid signaling has been found in pigs, horses, cattle, sheep, and dogs (Schjenken & Robertson, 2014).

This process of seminal activation results in the production of several different types of immune cells that aid in successful pregnancy. Cytokines are one of these types of immune cells, and they are responsible for organizing cell-to-cell activity via cellular signaling (Robertson, 2005). Once the production of cytokines has been induced by seminal exposure, they are secreted into the fluid of the uterus to target the cytokine receptors on the embryo. Once this activity begins in the uterus, these cytokines express their embryotrophic properties by organizing a nourishing environment in the oviduct and uterus for the embryo to avoid embryonic loss (Robertson, 2005). One important cytokine, in particular, is transforming growth factor β (TGFβ). Fair (2015) stated that the TGFβ found in seminal plasma is the primary trigger for the inflammatory response that occurs at semen exposure. While inside the male it is thought to be in a latent form, which makes the transition to an active form by enzymes such as plasmin to induce a

leucocytic response in the female reproductive tract (Fair, 2015; Schjenken & Robertson, 2014). According to Schjenken and Robertson (2014), both paternal antigens and cytokines play key roles in the signaling events that result in immunological tolerance of the conceptus.

Seminal activation also contributes to the production of regulatory T cells. Regulatory T cells, otherwise known as Treg cells, function to suppress cytokine production as well as effector function of B and T cells, dendritic cells, macrophages and natural killer cells in order to avoid the harmful effects of destructive immunity (Robertson et al., 2009; Schjenken & Robertson, 2014). Treg cells are found throughout the body, including the gut where inflammation exacerbates any problems already present. In this case,Treg cells are found in the uterus and play a large role in the success of a pregnancy. These specialized cells must be activated in order to suppress inflammation and protect the fetus from being subjected to a harsh immune response. Both the developing conceptus and sperm express antigens that would typically be recognized by the immune system as foreign and could induce an inflammatory immune response that could terminate the pregnancy. Instead, under normal circumstances, peripheral tissues are induced to produce Treg cells when reproductive antigens are detected (Robertson et al., 2009). The job of the Treg cells is to recognize that these cells are indeed foreign, but are not harmful and do not need to be acted against, which is a job that no other cell has the specific capacity to do. This idea is known as is known as Treg cell-mediated immune tolerance, or in this case, maternal tolerance. In mice, if the mother is deficient of Treg cells, the allogenic fetus will be rejected (Aluvihare et al., 2009).

In Robertson's lecture (2015), she discussed the main factors that were important in establishing a sufficient Treg cell population. In addition to the proper phenotype of dendritic cells and endometrial macrophages, a balance of cytokines, and proper estrogen and progesterone levels, it is also important that the seminal fluid that the female is exposed to contains MHC antigens and immune suppression agents (Robertson, 2015). According to Leung et al. (2000), the precise mechanism to which maternal tolerance occurs is not thoroughly understood, however it is likely that these MHC antigens are changed and immunomodulatory signals are produced in order to establish maternal tolerance. The research of Schjenken and Robertson (2014) supports this claim, stating that it is likely genetic mismatch of MHC antigens that stimulated an increase in the Treg cell population.

When observing the lymph nodes that are responsible for draining the uterus, Aluvihare et al., (2004) reported that the number of Treg cells had already increased two days after mating. Additionally, if the Treg cell level is depleted two days after mating or Treg cells do not function properly, implantation failure is likely to occur (Zenclussen et al., 2005). This illustrates the importance for Treg cells prior to implantation. It is thought that the exposure of Treg cells to seminal plasma during mating is an important step in activating the Treg cells to protect the growing fetus in the future by maintaining an immunologically suppressed uterine environment (Robertson et al., 2009). There are two times when the Treg cells are exposed to paternal alloantigen during reproduction. The first is when the female is exposed to seminal fluid during mating, and the last is during the process of embryo implantation (Robertson et al., 2009). It is known that Treg cells

play a large role in the embryo implantation process, therefore Robertson et al. (2009) hypothesized that the male alloantigens that are in seminal fluid might contribute to the priming of regulatory T cells prior to implantation. Through the research of Robertson et al., (2009) it was found that the plasma component of the semen as well as the sperm are necessary to activate the T cells in the lymph nodes in contact with the uterus maternal tolerance. The general mechanism of activation of Treg cells in the uterine lymph node is as follows. First, the exposure of the female to seminal fluid promotes recruitment and maturation of tolerogenic dendritic cells that function to phagocytose sperm and male somatic cells and bring them to the draining uterine lymph nodes (Robertson et al., 2009). Then, the sperm and male somatic cells interact with Treg cells in the uterine tissue, which activates Treg cells that express T cell receptors that react with antigens in seminal fluid (Robertson et al., 2009). The activation of Treg cells induces proliferation of cell numbers, which explains why the Treg cell numbers rise as early as two days after mating in mice.

In addition to the immune cell types discussed, there are countless other factors that contribute to reproductive success, reinstating why a delicate balance is so important during pregnancy. Not only do components of the seminal plasma stimulate a leucocytic response themselves, but they also associate with the endometrium to produce additional molecules that contribute to the overall immune response. Bacterial lipopolysaccharide, Toll-like receptors and many other factors that were not discussed play a role in establishing a uterine environment that is optimal for a healthy pregnancy (Schjenken & Robertson, 2014).

Analysis of Thesis Research

This particular study was performed by first collecting peripheral blood mononuclear cells from three different cows. The peripheral blood mononuclear cells were split into two cultures, with one culture containing IFN and one culture not containing IFN. After 24 hours, the peripheral blood mononuclear cells were added to immortalized bovine endometrial cells and cultured for 24 hours. The bEND cell line was used, which simply means that the cells were from endometrial tissue of female cows. The control of the experiment was just the bovine endometrial cells.

	Fold Change
CONT	1.4
IFN	212
PBMC	1.2
PBMC + IFN	1.3

Figure 1: Fold change values of the control sample (bEND cells), interferon sample, peripheral blood mononuclear cell (PBMC) sample, and the combined PBMC and interferon sample.

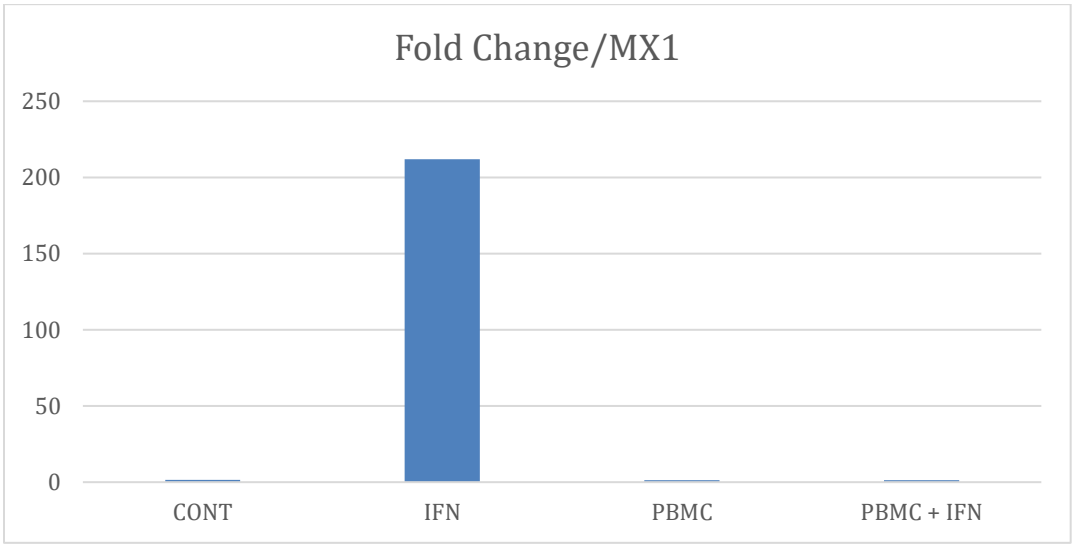

Figure 2: The fold change samples in the Figure 1 chart were put on a bar graph to illustrate the drastic fold increase in the fold change of IFN, showing that IFN is stimulating the Interferon stimulated gene, MX1.

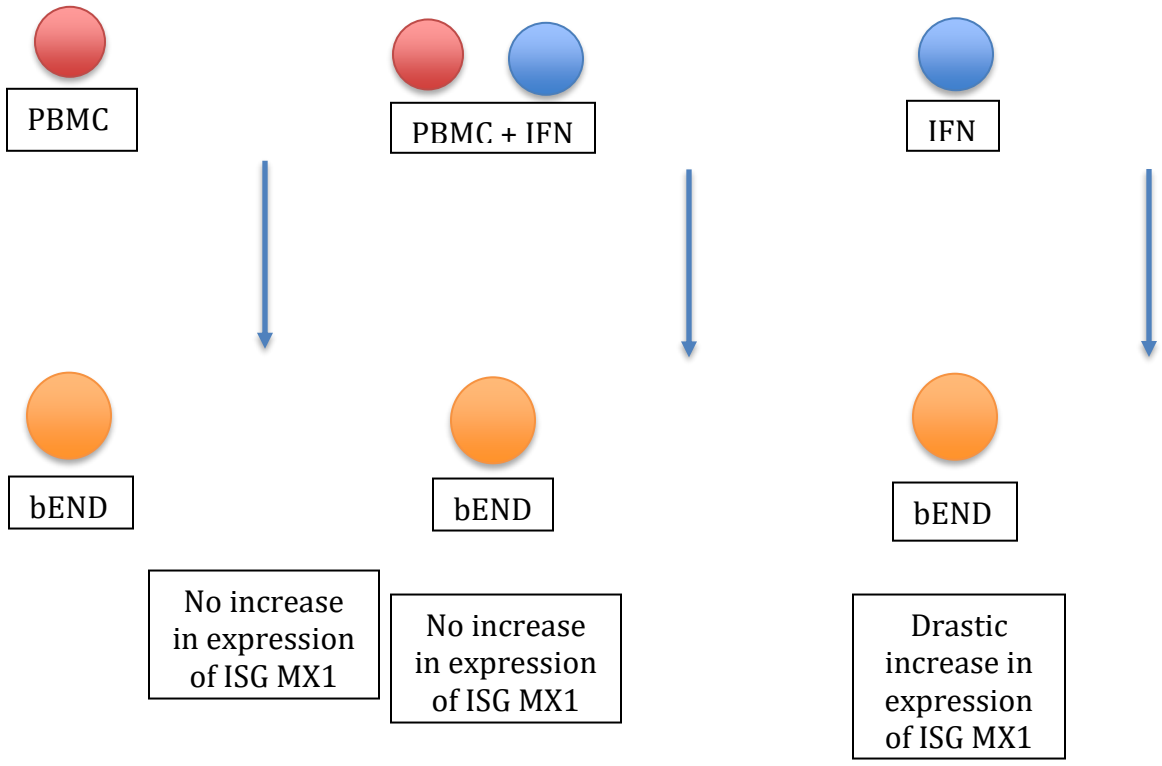

Figure 3: This visual representation shows that when bovine endometrial cells are exposed to peripheral blood mononuclear cells, a pathway that is separate from the interferon pathway is stimulated.

Discussion

The particular gene of focus in this research was MX1, an interferon-stimulated gene that is stimulated by Type I and Type III IFN to induce an antiviral response. As previously discussed, these types of responses are crucial for a healthy pregnancy by protecting the fetus from infection. The results of the research of Ideta et al. (2006) indicated that peripheral blood mononuclear cells stimulated a response in the endometrial tissue, and the results of this research narrowed the response down by verifying that PBMCs stimulate a non-interferon pathway in the bovine endometrial cells. This was indicated in both Figures 1 and 2. The fold change of the bEND cells themselves (control) was 1.4 in comparison to the interferon stimulated gene (ISG) MX1, showing that the endometrial cells do not stimulate ISGs themselves. The fold change of interferon and bEND cells was 212 in comparison to MX1, which was expected because MX1 is an interferon-stimulated gene. The fold change of PBMCs and bEND cells was 1.2 in comparison to MX1 showing that there was almost no increase in expression of MX1 when PBMCs and bEND cells were co-cultured together. The fold change of the co-culture of PBMCS, IFN and bEND cells in comparison to MX1 was 1.3 which showed that there was almost no increase in expression of MX1 in this case either. With these values, it was concluded that the PBMCs stimulated a non-interferon pathway in the bovine endometrial cells. A fold change value of ~1 indicates that there was no change in gene expression. In order to

conclude that the PBMCs were, in fact, stimulating an interferon pathway in the bEND cells, the fold change should be much higher than 1.3, like in the case of the IFN culture. From this, we can conclude that when bovine endometrial cells were exposed to PBMCs, the PBMCs did not act as IFN tau, and a pathway that is currently unknown was stimulated in order to promote successful pregnancy.

Recently, Chase et al. (2015), found that when they primed peripheral blood mononuclear cells with Interferon tau and transferred them into the uterus, pregnancy rates were increased. In order for the embryo to survive in the uterus, it must begin to secrete IFN tau in order to inhibit luteolysis. This particular research showed that the immune cells are not necessarily associated with IFN tau. The conclusion that formed was that IFN tau interacts with the embryo, and immune cells are doing something else at the same time.

Future research is required to identify the pathway that the immune cells (PBMCs) stimulate in the bovine endometrium. When this pathway is discovered, further assessment has the potential to identify a role that this pathway plays in the rate of embryonic survival. As discussed earlier, seminal priming of immune cells has been found to play a role in the rate of embryonic survival and implantation. Therefore, it would be beneficial for future researchers to look at the role that immunological priming of the PBMCs plays in the success of this unknown pathway in the bovine endometrial cells that is stimulated by the PBMCs. Further research into this unknown pathway, as well as reproductive immunology as a whole, has potential to help producers increase pregnancy rates in their cattle by allowing them to both understand and address embryonic loss in a new way.

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