Lactoferrin affects fertilization, implantation, and pregnancy in rats

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Research Article

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Abstract

Lactoferrin (LF) is present in the oviduct, reduces *in vitro* gamete interaction, and affects sperm capacitation parameters in humans. Our aim was to investigate LF actions on further stages of the reproductive process in the Wistar rat model. Motile sperm were obtained from cauda epididymis to assess LF binding by direct immunofluorescence and LF effect on acrosome reaction (AR) using a Coomassie blue staining. After ovarian hyperstimulation of female rats, oocytes were surgically recovered and coincubated with motile sperm and different doses of LF to estimate the *in vitro* fertilization (IVF) rate. To evaluate the LF effect on pregnancy and embryo implantation, female rats (80 days old) were placed with males and received daily intraperitoneal injections of LF during one complete estrous cycle (pregnancy experiments) or during the first 8 gestational days (implantation experiments). The number of pregnant females and live born pups was recorded after labor. Moreover, the number of implantation sites was registered during the implantation period. LF was able to bind to the sperm head, midpiece, and tail. Ten and 100 µg/ml LF stimulated the AR but reduced the IVF rate. The administration of 100 and 200 mg/kg LF significantly decreased the number of implantation sites and the litter size, whereas 100 mg/kg LF declined the pregnancy rate.

The results suggest that LF might interfere with the reproductive process, possibly interfering with gamete interaction or inducing a premature AR; nevertheless, the mechanisms involved are yet to be elucidated.

Introduction

Mammalian fertilization is a complex process that takes place in the oviduct. Sperm cells pass through the female reproductive tract and move ahead to the fertilization site while undergoing physiological modifications, known as sperm capacitation. Capacitation is critical for the success of fertilization, and it involves hyperactivation of the flagellum and the acrosome reaction (AR) (Hernández-González et al., 2006; Arcelay et al., 2008; Krapf et al., 2010; Visconti et al., 2011; Stival et al., 2016).

It has been shown that the oviduct contributes to the selection of spermatozoa before fertilization and that it has an active role in modulating the sperm capacitation (Mahé et al., 2021). The oviductal tissue secretes several proteins that could modulate gamete function and sperm-oocyte interaction (Gadella, 2013; Ghersevich et al., 2015; Massa and Ghersevich, 2019).

Previously, we isolated and identified lactoferrin (LF) in the human oviductal tissue and secretion (Zumoffen et al., 2013). LF is a 79 kDa glycoprotein that contains iron binding sites and is widely expressed in several tissues such as respiratory tract epithelium and urogenital mucosa (Levay & Viljoen, 1995; Brock, 2002; Ward et al., 2005). This protein can bind to different membrane receptors, which may activate distinct signaling pathways, or allow the internalization of LF, which appears to be involved in modulating the transcription of DNA sequences (Legrand et al., 2005; Vogel., 2012; Ghersevich et al., 2017).

It is well known that LF participates in the modulation of the innate and acquired immune system (Latorre et al. 2012; Legrand 2012; Pang et al. 2014; Kanwar et al. 2015). The concentration of LF in the blood stream can increase several-fold during inflammatory processes and sepsis (Berkestedt et al., 2010; García-Montoya et al., 2012).

We have reported that LF expression varies in the human oviduct during the ovarian cycle, being highest around the periovulatory phase (Zumoffen et al., 2013). The fact that LF gene expression is responsive to estrogens could explain this finding (Liu & Teng, 1992; Teng, 2002). Our previous results also indicated that the presence of LF enhances the induced AR and the protein tyrosine phosphorylation of human spermatozoa (Zumoffen et al. 2015).

We also observed that LF was able to bind to the human gametes and to decrease the human gamete interaction *in vitro* in a dose-dependent manner (Zumoffen et al. 2013, 2015). The presence of LF decreased the availability of sperm α-D-mannose receptors which have been involved in gamete interaction (Hershlag et al. 1998; Zumoffen et al. 2013, 2015). Furthermore, a recent study showed that the rate of *in vitro* fertilization (IVF) was associated with the levels of LF in the cervical secretion of patients undergoing assisted reproduction treatments (Massa et al. 2021).

Considering that LF affected sperm function parameters and gamete interaction *in vitro*, we evaluated the effect of this protein on advanced stages of the reproductive process, such as fertilization, embryo implantation, and pregnancy in an animal model, both *in vivo* and *in vitro*. The rat model has been widely used to evaluate LF actions (Tsubota et al. 2008; Onal et al. 2010; Hegazy et al. 2016) because of being an animal of short ovarian cycle and about 3 weeks of gestation, which allows an adequate experimental design and follow-up. In addition, LF expression in the rat reproductive tract is well documented (Teng et al. 2002).

Materials And Methods

Compliance with Ethical Standards

This research was approved by the Institutional Board of Care and Use of Laboratory Animals of the Facultad de Ciencias Bioquímicas y Farmacéuticas (Universidad Nacional de Rosario).

Animals

Adult male and female Wistar rats were kept under 12 h light/12 h darkness regimen, regulated temperature, and free access to water and food during the experiments.

Chemicals and reagents

Unless otherwise mentioned, all the chemical products and reagents were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA) and were of the highest purity available.

Acrosome reaction assessment

Male Wistar rats (100-140 days) were euthanized and motile spermatozoa were collected from the epididymal cauda. Several cuts were made on the testicular cauda and spermatozoa were collected by swim out in HTF medium at 37ºC and 5% pCO_2 for 15 min. The sperm concentration was estimated with a Neubauer chamber and adjusted to 1.5 x 10^6 sperm/ml. Sperm cells were incubated under capacitating conditions in the absence (controls) and presence of 10 and 100 µg/ml human milk LF. Cultures were performed in HTF medium supplemented with 5 mg/ml human serum albumin (HSA, Irvine Scientific, Santa Ana, CA, USA) during 3h at 37°C and 5% CO_2. Two aliquots from each treatment were either incubated during 30 min in the absence (spontaneous AR) or presence of 20 µM progesterone (induced AR). Finally, aliquots of sperm suspensions were seeded onto a microscope slide, air dried, fixed with paraformaldehyde (4 % w/v) for 1 h, and then stained with Coomassie Blue (0.22 % w/v) (Bendahmane et al. 2002). Spermatozoa were examined at a magnification of 200X under a light field microscope and the percentage of acrosome-reacted sperm was determined. The inducible population (% IP) was calculated as: % acrosome-reacted cells in induced AR with progesterone minus % acrosome-reacted cells in basal AR. The results were reported as the mean ± standard error of the mean (SEM) of eight experiments.

Binding of LF to rat spermatozoa

The experiments were performed with LF and BSA, both conjugated with fluorescein isothiocyanate (FITC). Briefly, 1 mg of protein was incubated in the presence of 50 µl of FITC (1 mg/ml in DMSO) for 8 h. The reaction was stopped with NH_4Cl and the unbound FITC was removed by dialysis (Zumoffen et al. 2013).

Rat epididymal cauda spermatozoa were obtained as previously mentioned. Spermatozoa (5 x 10^6/ml) were incubated under capacitating conditions in HTF medium supplemented with 5 mg/ml HSA (HTF + HSA), in the absence or presence of LF-FITC (100 µg/ml) or BSA-FITC (100 µg/ml) at 37ºC, 5% pCO_2 for 1 h. After incubation, spermatozoa were washed twice with PBS and centrifuged at 4000 rpm for 5 min. Finally, cells were spread out on slides, air-dried, fixed with paraformaldehyde solution (2% w/v), and washed again with PBS. The cells were mounted with glycerol:PBS (9:1) and observed under an epifluorescence microscope (Primo Star, Zeiss, Oberkochen, Germany).

In vitro fertilization

Hyperovulation was stimulated in 80 days-old female rats with 50 IU of pregnant mare serum gonadotropin (PMSG, Zoetis, Argentina) and after 50 h they were further treated with 50 IU of human chorionic gonadotropin (hCG, Syntex, Argentina). Fifteen hours later, the animals were euthanized and the oocytes were retrieved from the ampullary region of the oviducts and morphologically classified; only the cells in metaphase II (that extruded the polar body) were used for fertilization experiments (Aktuğ et al., 2013).

Motile sperm were collected from the epididymal cauda as described above and were incubated under capacitating conditions (HTF + HSA) at 37°C and 5 % CO_2 for 3 h. To carry out the IVF experiments, three or four oocytes were placed on drops of HTF+HSA in the absence (controls) or presence of LF (1, 10 o 100 µg/ml) and then inseminated with $1x10^5$ spermatozoa/ml and coincubated for 9 h (37°C, 5 % CO_2). Finally, oocytes were stained with Hoechst 33258 (Invitrogen, Waltham, MA, USA) and examined at 100X magnification under a fluorescence microscope (Nikon Eclipse TE-2000-E2). Fertilized oocytes were detected by observation of the presence of two pronucleus. The rate of IVF (%) was estimated considering the number of produced embryos regarding the number of inseminated oocytes. Data were expressed as the mean ± SEM of nine experiments.

Effect of LF on pregnancy

The rat model has been widely used to study *in vivo* actions of LF (Tsubota et al., 2008; Onal et al., 2010; Hegazy et al., 2016). In addition, the rat possesses a short ovarian cycle and about 3 weeks of gestation, which allows an adequate experimental design and follow-up. Furthermore, LF expression in the rat reproductive tract has been well documented (Teng 2002).

Female adult Wistar rats were daily subjected to the determination of the phase of estrus cycle by assessing the vaginal cytology (Paccola et al., 2013). After two complete natural estrus cycles, at least five animals were randomly assigned to each study group and were treated daily during one complete estrus cycle with an intraperitoneal (i.p.) injection either of 0.9 % w/v NaCl (controls), 100 mg LF/kg, 200 mg LF/kg, or 400 mg LF/kg of body weight (Fig 1a). On proestrus day, females were mated with a male. After that, rat gestation was followed up by weight control for 21 days or until labor, and pregnancy was registered. After delivery, the litter size was recorded and the rats were euthanized. The uterine horns of the mother rats were recovered and the number of implantation sites was registered, as well as the litter size.

Effect of LF on embryo implantation

Eighty days-old female Wistar rats were cycled by vaginal cytology (Paccola et al. 2013) to determine the phase of the ovarian cycle during two complete estrus cycles. On proestrus phase of the following cycle, female rats were mated with male rats until the next diestrus stage. Subsequently, female rats were randomly assigned to four groups (at least five animals per group) and were given a daily i.p. injection of either 50 mg LF/kg, 100 mg LF/kg, 200 mg LF/kg or 0.9% w/v NaCl (controls) during eight days. Thereafter, animals were euthanized and uterine horns were recovered. The number of implantation sites was registered (Fig. 1b).

Statistical analysis

Analysis of variance (ANOVA) followed by a parametric or non-parametric test was used to compare the results of each experimental group. A $p<0.05$ was considered significant.

Results

Localization of LF binding sites on rat spermatozoa

LF binding to rat spermatozoa was evaluated by direct immunofluorescence. Microscopic analysis revealed that LF was able to bind to most rat spermatozoa. The fluorescent staining was localized on the sperm head, intermediate piece, and tail (Fig 2a). The control staining performed with spermatozoa incubated in the absence of LF-FITC or in the presence of BSA-FITC did not show a fluorescent staining (Fig 2c).

Effect of LF on AR

We studied the effect of LF on rat sperm spontaneous and induced AR. The observations indicated that the % of acrosome-reacted sperm was significantly increased in the presence of LF respect to controls, as shown in Fig 3b.

However, the presence of LF did not affect the induced AR, as we observed that the mean IP was not significantly different from the IP in the 10 μg/ml LF nor in the 100 μg/ml LF treatments.

Effect of LF on the IVF rate in Wistar rats

The IVF rate was evaluated in rat gametes coincubated with or without increasing doses of LF. The presence of LF caused a dose-dependent decrease of the IVF rate (Fig 4). The mean IVF rate values were: 68.7 ± 5.2 % (control group), 51.7 ± 10.4% (1 μg/ml LF), 41.0 ± 6.6 % (10 μg/ml LF), and 31.4 ± 6.4% (100 μg/ml LF).

Effect of LF on pregnancy rate and litter size

Female rats (n=25) were randomly assigned to one of four groups, receiving a daily i.p. injection of the corresponding treatment (0, 100, 200 or 400 mg LF/kg) during an entire estrus cycle until the diestrus of following cycle. At day of proestrus, the rats were mated with males. The pregnancy rates from the different treatments are shown in table 1.

The % of pregnant animals was reduced when the females were treated with the doses 100 mg LF/kg and 400 mg LF/kg. In rats treated with 100 mg LF/kg, the pregnancy rate was significantly lower than in control group.

The treatments with 100 mg LF/kg and 200 mg LF/kg significantly decreased the average number of offspring born respect to the controls. The mean number of born live pups considering all females rats that received LF treatment was lower than in controls.

	Control	100 mg LF/kg	200 mg LF/kg	400 mg LF/kg
Pregnancy rate (%)	83.3 ± 8,3[a]	45.8 ± 4.1[a]	85.7 ± 9.2	58.3 ± 9.4
Average number of live pups	9.8 ± 1.0 [b,c]	4,8 ± 0,5[b]	6,4 ± 1,5[c]	7,3 ±1,3

Table 1 Assessment of the effect of LF on pregnancy rate and litter size. a p< 0.05; b: p<0.05; c: p<0.05.

Interestingly, the uterus of some of the animals treated with LF, showed a marked asymmetry in the distribution of implantation sites, showing one of the uterine horns with few or no implantation sites and the contralateral horn with several sites.

LF effect on embryo implantation

Twenty-four female rats were mated with males and randomly assigned to one of four groups (control, 50, 100, and 200 mg LF/kg), receiving a daily i.p. injection of the corresponding treatment during the first 8 gestational days. After that, the animals were euthanized and uterine horns were recovered. The mean number of implantation sites registered in each treatment group was: 13.0 ± 1.4 in the control group, 11.6 ± 2.9 in the group treated with 50 mg LF/kg, and 1,4 ± 1,0 in the group treated with 200 mg LF/kg; while in the group of rats that received 100 mg LF/kg no implantation sites were detected.

To analyze the data, the mean number of implantation sites of control was arbitrarily considered as 100%. Then, the % of implantation relative to the control was calculated for each group. The results indicated that the number of implanted embryos was significantly reduced after the administration of 100 mg LF/kg ($p<0.001$) and 200 mg LF/kg ($p<0.001$) (Fig 5a). The average number of implantation sites in animals treated with the dose of 50 mg LF/kg was not significantly different respect to that in controls, while it was significantly higher when compared to that of rats treated with the higher doses of LF ($p<0.01$).

Discussion

In mammals, the oviductal environment plays a key role in sperm selection and capacitation, and the oviduct proteins contribute to the modulation of the physiological processes that allow fertilization and early embryo development (Massa and Ghersevich 2019; Mahé et al., 2021).

Previous studies carried out in our laboratory detected the presence of LF in the human oviduct and in its secretion. In addition, previous results indicated that LF affects both sperm functional parameters and the interaction between human gametes *in vitro* (Zumoffen et al., 2013, 2015). Another report by Kobayashi et al., (2021) observed that LF modulated bovine sperm motility and viability and promoted sperm capacitation-dependent changes *in vitro*. Based on previous findings, the present work examined the effects of LF in later stages of the reproductive process, such as fertilization and implantation, using the rat as an experimental model.

It is accepted that the ability of spermatozoa to react to an AR inducer is an indirect indicator of their capacitation status (Varano et al., 2008; Ickowicz et al., 2012; Zumoffen et al., 2015). The results indicated that certain doses of LF were able to induce the basal AR in rat spermatozoa, but did not affect progesterone-induced AR. It is interesting to mention an apparent difference between species since a previous study showed that LF was able to stimulate follicular fluid-induced AR but it did not affect spontaneous AR in human spermatozoa (Zumoffen et al., 2015). The fact that sugar moieties in LF play a crucial role in the interaction with cell receptors could reflect the different effects of the protein when it is assessed in different species (Zimecki et al., 1991). It should be noticed that in previous studies LF concentrations up to 100 µg/ml did not affect sperm viability and motility of human spermatozoa (Zumoffen et al., 2015). On the other hand, the incubation of rat spermatozoa in the presence of 100 µg/ml of LF (the maximum concentration used) did not affect sperm viability (data not shown) suggesting that the reported effect of LF on basal AR would not be due to a cytotoxic effect. Considering the fact that LF stimulated phosphorylation pathways in human spermatozoa and that at least one sperm receptor for the protein has been reported, it is likely that the LF-induced AR in rat spermatozoa could result from the action of LF mediated by specific receptors (Wang et al., 2011; Zumoffen et al., 2015). This hypothesis is supported by the finding that LF can bind to rat spermatozoa, as was previously observed in human spermatozoa (Zumoffen et al., 2013).

The present study showed that certain doses of LF affected the fertilization and the implantation processes in rats. The results of the IVF experiments indicated that the presence of increasing concentrations of LF produced a dose-dependent decrease in the IVF rate. The effect was significant when gametes were coincubated with the same doses of LF that caused an increase in AR. It has been suggested that the AR should occur near the oocyte to achieve a successful gamete interaction and fertilization (Avrech et al., 1997; Jin et al., 2011; Hirohashi and Yanagimachi 2018). Thus, the decrease in the IVF rate in the presence of LF could reflect a higher number of spermatozoa that underwent a premature AR, decreasing the chance of fertilization. This result is consistent with our previous work that showed that the interaction between human gametes *in vitro* was decreased by the presence of LF (Zumoffen et al., 2015).

The results of the embryo implantation study in female rats treated with LF during 8 days after mating with a male indicated that the administration of 100 mg/kg and 200 mg/kg of LF was associated with a marked decrease in the number of implanted embryos. Interestingly, a dose 100 mg/kg of LF also significantly reduced the pregnancy rate while the treatment with 100 mg/kg or 200 mg/kg significantly decreased the number of offspring respect to controls in treated rats *in vivo*.

In addition, the observation of the uterus from LF treated animals after delivery revealed that some of them presented a marked asymmetry in the number of implantation sites, showing one of the uterine horns with few or no placentation sites and the contralateral one with several sites.

The present observations increase the evidence on the effects of LF on fertility parameters.

Among the effects of LF reported by other authors, is the ability to interfere with the interaction between certain microorganisms and their receptor molecules in target cells, as well as to modulate the response of immune cells (Maritati et al., 2017; Lepanto et al., 2019; Presti et al., 2021). It has been shown that LF was able to increase the percentage of natural killer cells, to modulate T and B cells, and antibody production (Artym et al., 2004). Several studies have reported that LF modulates the levels of certain cytokines, both anti-inflammatory and pro-inflammatory, and increases the expression of the colony-stimulating factor of granulocytes and macrophages (Baveye et al. 1999; Legrand et al. 2004; Actor et al. 2009; Legrand 2016; Presti et al. 2021).

In addition, previous studies found that LF favors the differentiation of CD4+ T lymphocytes with a rise in the Th1 / Th2 cytokine ratio, increasing the Th1-type immune response and the expression of Th1 cytokines (de la Rosa et al., 2008; Hung et al., 2010).

Taking into account these references and considering the results of our studies, we could hypothesize that at least part of the LF effect on gamete interaction and fertilization might result of an interference with the receptor-mediated recognition between gametes (Elass-Rochard et al., 1998; Valenti and Antonini, 2005; Kell et al., 2020). In this regard, the presence of LF was shown to reduce the availability of sperm mannose binding sites, which are associated with gamete recognition (Zumoffen et al., 2015).

On the other hand, it could be thought that the effect of LF on the immune cells could interfere with the success of embryo implantation, which depends on a receptive uterine immune environment during the interaction of the embryo and the endometrium (Mrozikiewicz et al. 2021).

It is known that infertility affects between 9 and 18% of the population and that there is a worldwide increase in the consultation rate on fertility-related problems (Chandra et al., 2005; Sun et al., 2019). It is estimated that in 15% of infertile couples the cause of infertility is unknown (Leaver, 2016). Thus, the study of factors involved in the modulation of the reproductive process could contribute to determine the causes of infertility in certain patients. In addition, it could facilitate diagnostic strategies and treatment tools for those couples who cannot conceive.

The results of the present work indicated that the presence of LF in certain concentrations, *in vitro* and *in vivo*, affected different stages of the reproductive process in rats, such as parameters of sperm capacitation, fertilization and embryo implantation. The results support the findings of our previous studies that indicated that the effects of LF on certain stages of the human reproductive process would also be dose-dependent (Zumoffen et al. 2013, 2015). LF concentrations in the female reproductive tract could fluctuate markedly at different stages of the ovulatory cycle (Teng, 2002; Zumoffen et al., 2013). Furthermore, inflammatory processes, which are frequent in the female reproductive tract, have been associated with marked increases of LF levels (Marey et al., 2016; Kruzel et al., 2017). Therefore, it could be thought that in certain situations, either physiological or pathological, changes in LF levels could influence the reproductive process, favoring it or perhaps interfering with it. Furthermore, in a recent study we reported that a higher level of LF in the cervical fluid of patients under IVF treatment were associated with lower IVF success rates (Massa et al., 2021).

In conclusion, the present study reveals effects of LF on different stages of the reproductive process. Nevertheless, further experiments are needed to investigate the mechanisms involved in the reported effects of LF, helping to determine potential uses of this protein for the control of the reproductive process or as a prognostic biomarker of fertilization success.

Declarations

Declarations

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Animals

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Funding

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Author contribution

EM, AG, MM, and MJM performed the experiments and analyzed the results. EM, FP and SG contributed to the experimental design, methodology, and analysis of the results. EM, AG, MM, and SG wrote the main manuscript and prepared the figures. FP and SG supervised the study and contributed to funding acquisition.

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Declarations

Conflicts of interest

The authors declare no conflict of interest.

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Figures

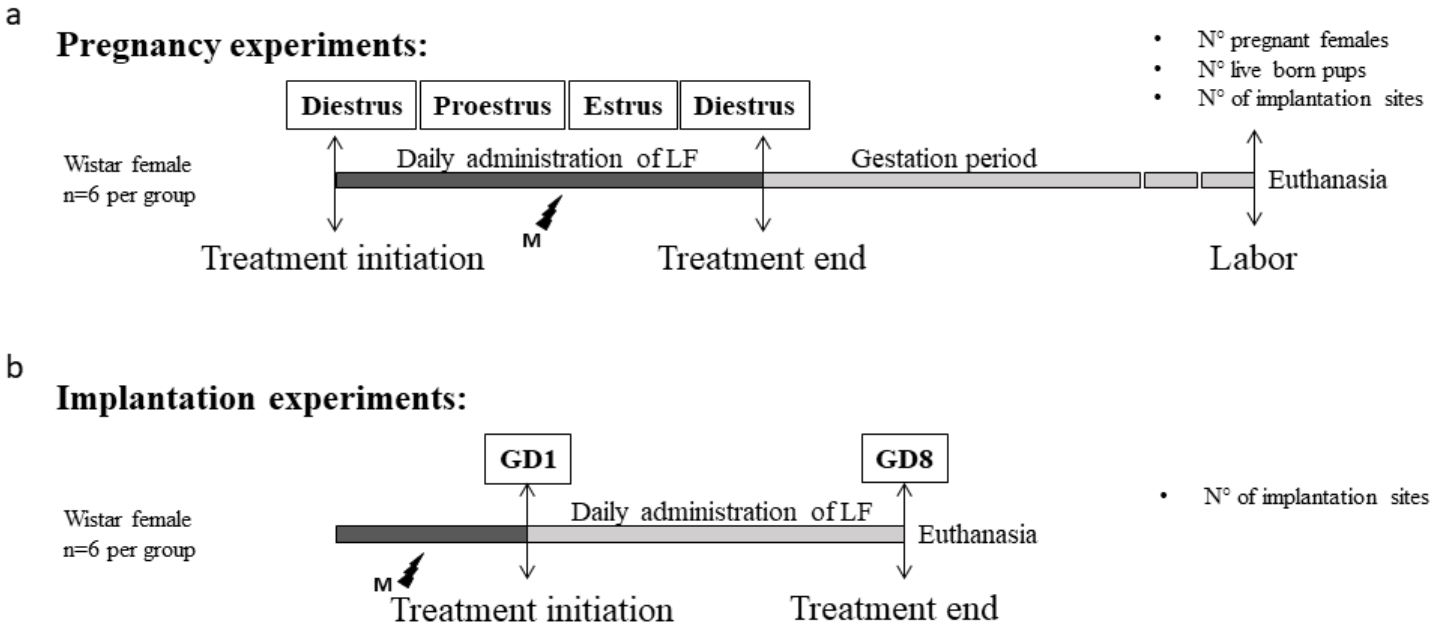

Figure 1

a) Experimental design of pregnancy and litter size assessment after LF treatment during a full estrus cycle in female Wistar rats. b) Experimental design of implantation sites evaluation after LF treatment during the first 8 gestational days in female Wistar rats. D=diestrus; P=proestrus; E=estrus; M=male rat; GD= gestational day.

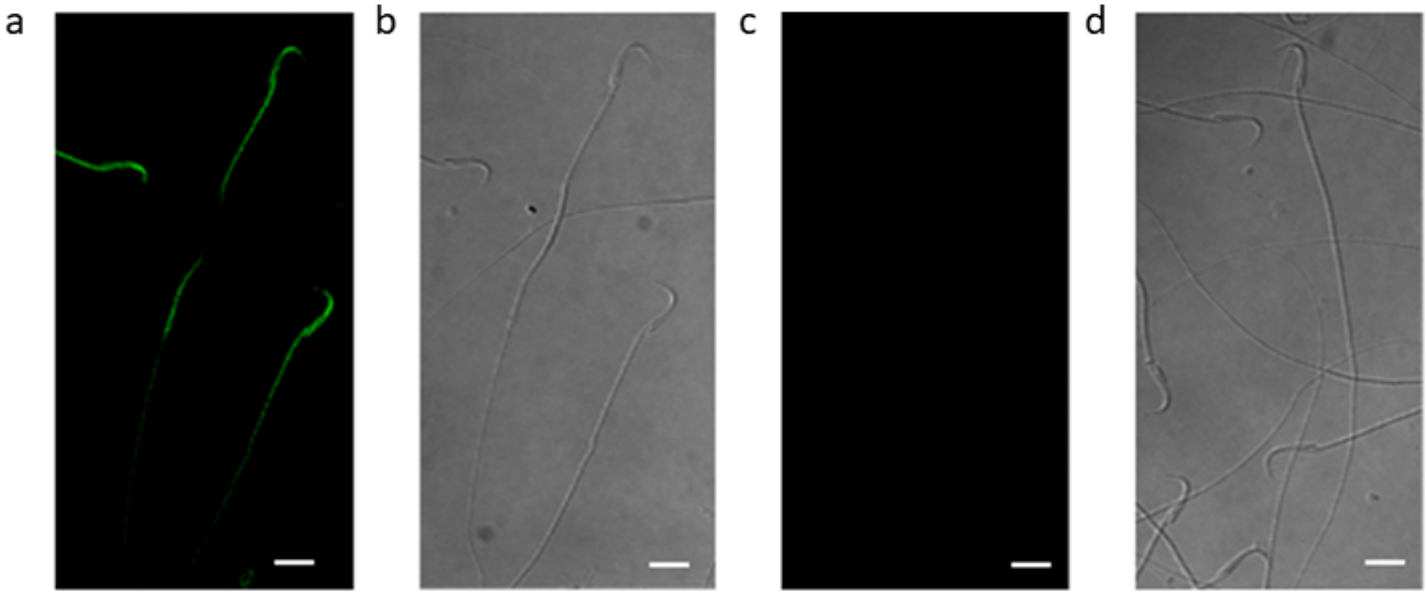

Figure 2

LF binding to rat spermatozoa. a) Fluorescence micrographs of rat sperm cells incubated with LF-FITC conjugate. b) Light field micrographs, same as in a. c) Fluorescence micrograph of sperm cells stained with BSA-FITC (negative control). d) Light field micrographs, same as in c. 100X augment. Bar= 10µm.

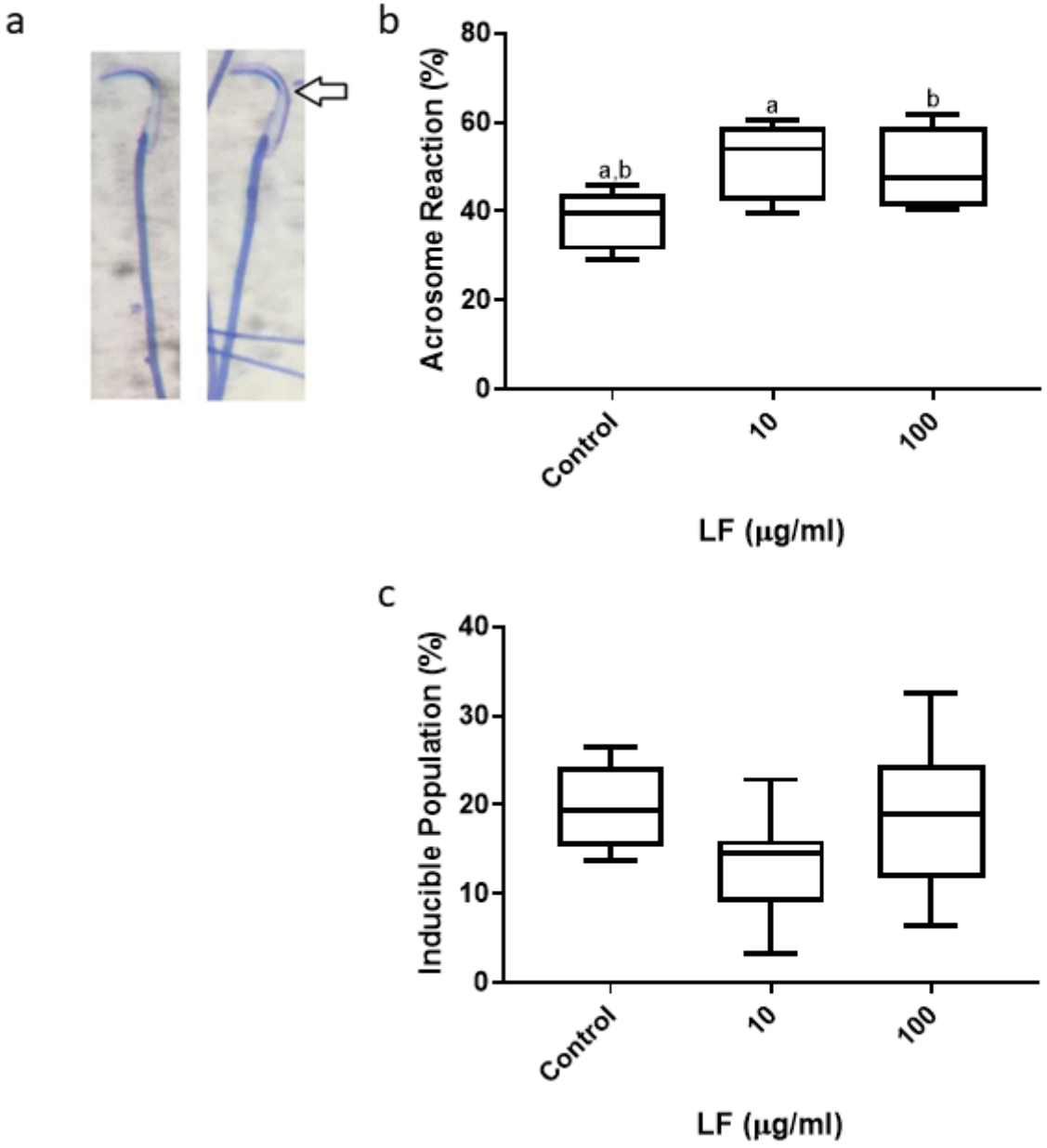

Figure 3

a) Rat spermatozoa stained with Coomassie blue stain. Left: spermatozoon without acrosome. Right: spermatozoon with an intact acrosome (indicated by the arrow). b) Box plot showing the percentage of basal AR in each group. a: $p<0.05$, b: $p<0.05$. c) Box plot showing the % of inducible population in each group. The inner line within the boxes corresponds to the median and the limits refer to 25 and 75 percentiles.

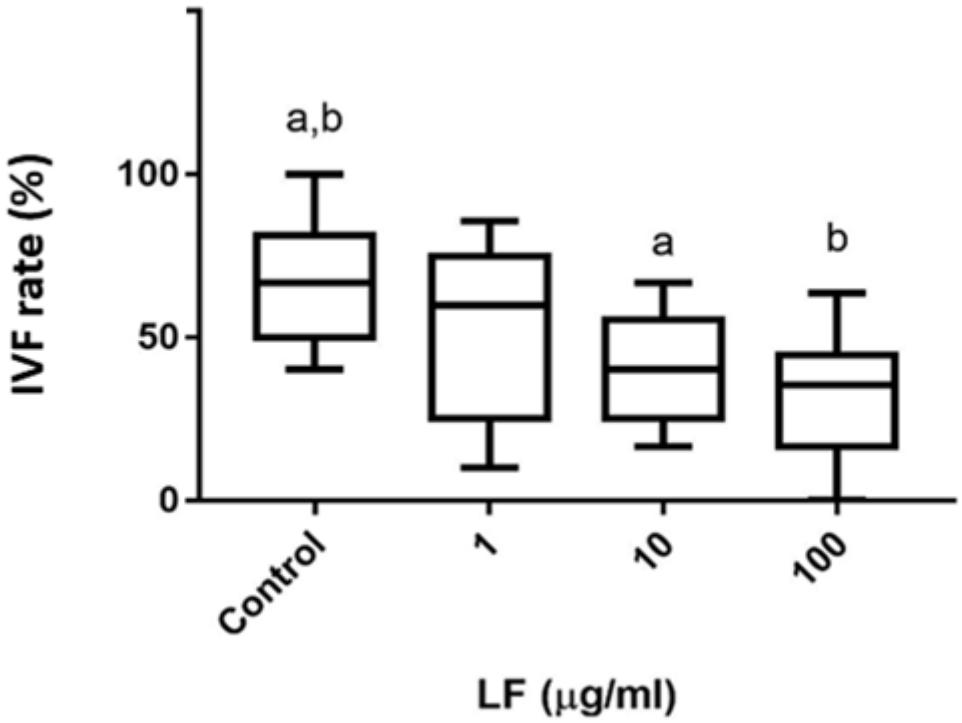

Figure 4

Box plot of the IVF rates reported in the rat gametes incubated with increasing doses of LF. The inner line within the boxes corresponds to the median and the limits refer to 25 and 75 percentiles. a: $p<0.05$, b: $p<0.001$.

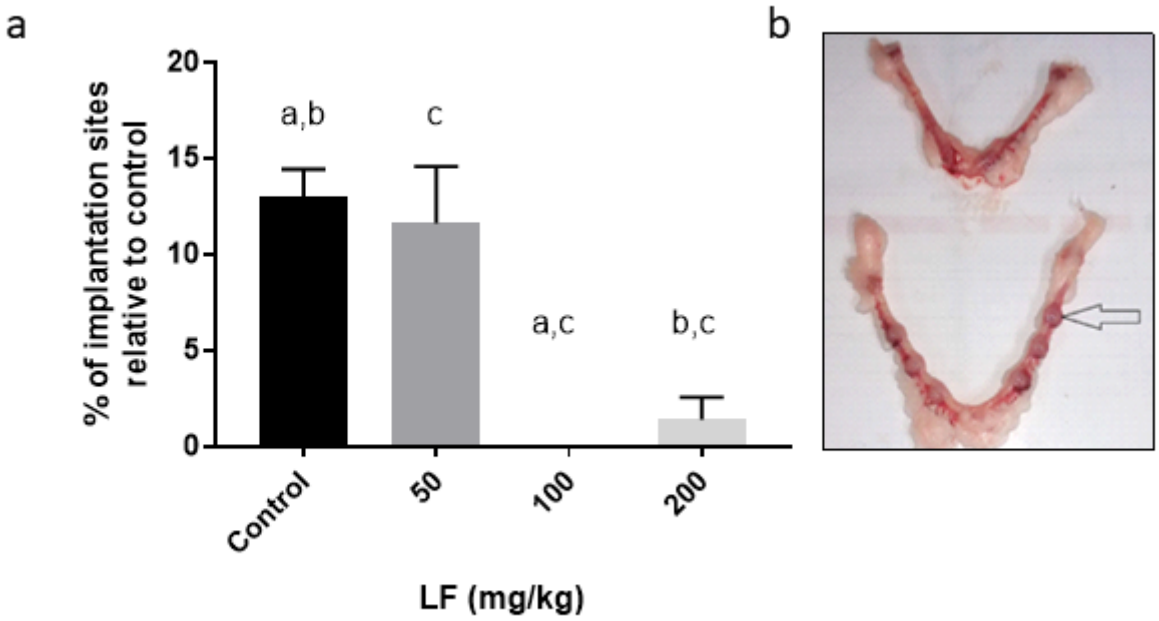

Figure 5

Effect of LF on embryo implantation. a) % of implantation sites detected in the different treatment groups (considering the control as 100%). a: $p<0.001$, b: $p<0.01$, c: $p<0.01$. b) Images of rat uterine horns from an animal treated with 100 mg LF/kg (above) and from a control animal (below). The arrowhead points at one implantation site.