In Vivo Evaluation of the Efficacy and Safety of a Novel Degradable Polymeric Film for the Prevention of Intrauterine Adhesions

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Conflict of interest statement

The authors (S.L., X.G., S.H., V.L.) currently have a patent for the polymer and its applications (WO2016020613). Two of the authors (S.H. and X.G.) have developed a start-up company based on the results of the current study, and one of the authors (S.L.)

is currently employed in this new company. The rest of the authors do not have any additional conflicts to disclose.

Abstract

Objective: To study the safety of DPF (degradable polymeric film) and its efficacy on reducing the risk of intrauterine adhesions (IUA) formation in a rat model.

Design: A series of Case control studies relying on random allocation where feasible.

Setting: University and good practice animal laboratories.

Animals: Animal models comprised female and male OFA (Oncins France Strain A) and female wistar rats.

Intervention(s) and measurements: OFA rats were used for in vivo evaluation of the impact of DPF on endometrial thickness and in vivo evaluation of the effect on fertility. For in vivo evaluation of the biological response, 40 wistar rats were randomly allocated to intervention and control groups with matched sampling time after surgery. Finally, for the in vivo evaluation of DPF efficacy on IUA prevention, a total of 24 wistar rats were divided into 3 groups that were treated with DPF, hyaluronic acid (HA) gel and a sham group.

Main results: DPF did not have a significant impact on endometrial thickness and no significant differences in the number of conceived or prematurely terminated pregnancies confirmed its non-inferiority to no treatment. DPF did not induce irritation at 5 and 28 days. Finally, DPF significantly reduced the likelihood of complete IUA formation compared to HA gel and sham implanted animals, where only 27% of the animals had their uterine cavity obliterated compared to 80% and 100% respectively.

Conclusion: DPF is a safe degradable polymeric film that is effective in preventing IUA formation after intrauterine curettage in rats.

Keywords: Adhesions; animal model; Intrauterine; medical device; Synechiae

Introduction

Intrauterine adhesions (IUA) result from an endometrial trauma leading to a partial or complete obliteration of the uterine cavity [1,2]. A Meta-analysis of 10 RCTs involving 912 women calculated an IUA prevalence following miscarriage of 19.1% (95% CI 12.8-27.5%) [3]. IUA can also occur after intrauterine surgical interventions for the management of uterine leiomyomas, septa and polyps. Diagnosis can be suspected when pain or abnormal bleeding occur after surgical or obstetrical intrauterine trauma [4]. Although the iatrogenic trauma is established as one of the main precipitating factors, the exact underlying pathophysiology of IUA formation is less well known. IUA can lead to primary or secondary subfertility, early or late miscarriage and increase the risk for several obstetric complications including abnormal placentation, postpartum hysterectomy, prematurity and cesarean section [3,5–8]

Hysteroscopy remains the mainstay for IUA diagnosis and treatment, nevertheless, carry the risk of IUA recurrence [9–11]. Several anti-adhesive strategies including hormonal therapy, intrauterine devices, intrauterine balloons and barrier gels have been evaluated with controversial results [12,13]. Although there is evidence that barrier gels can reduce the incidence and severity of de novo IUA after operative hysteroscopy [13], there is limited evidence relating to their effectiveness on improving pregnancy rates [14]. We previously described the efficacy of a degradable polymeric film (DPF), in a rat model, on post-operative abdominal adhesion [15,16]. The aim of the present study was to assess the safety of DPF, its efficacy on reducing the risk of IUA formation and impact on fertility in a rat model.

Material and methods

Polymer synthesis and shaping

DPF was synthesized at the biopolymer laboratory of Montpellier, France, and previously described by Leprince et al. [16]. For the evaluation of endometrial thickness and fertility impact, DPF was shaped by hot extrusion with a Noztek Powder Filament Extruder (Shoreham, England) to obtain a polymer filament with a diameter of 1.5 mm. For the evaluation of biological response and the evaluation of IUA prevention, DPF was synthesized at Polymer Expert (Pessac, France) and shaped by hot-pressure to obtain a film (10 mg; length 1.3 +/-0.1 cm). The co-polymer was sterilized by irradiation between 27.7 and 36.6 kGy by Ionisos (Chaumesnil, France). DPF is now manufactured under the brand name of "Womed Leaf".

Animal preparation

The evaluation of endometrial thickness and fertility were conducted at the Experimental Department of University of Montpellier (Montpellier, France) on OFA (Oncins France Strain A) female rats (7-8 weeks old) weighing between 250 and 275 grams. OFA male rats weighing between 275 and 300 grams (purchased from Earl Cegav SSC, St Mars d'Egrenne, France) were also required for the fertility experiment. While the evaluation of biological response and the evaluation of IUA prevention were conducted at Phycher Bio Development (Pessac, France), under good laboratory practice, on female wistar rats (SPF Caw; 9-10 weeks old), supplied by Janvier Labs. The animal strain used depended on availability at the time of conducting the experiment. All animal investigations were approved by the Ethics Committee of the French Ministry of Education and Research (contract number 02367.01, task order 1065 and EC N°.76) and carried out in accordance with the EU Directive 2010/63/EU for animal experiments. All efforts were made to minimize animal suffering or distress, and to use the minimum number of animals

102 necessary to produce reliable scientific data. All the animals were in quarantine for one
103 week prior to treatment. They were placed individually in an air-conditioned animal
104 holding facility (22°C +/- 3°C; 30%-70% humidity) with free access to food (SAFE®)
105 and water. They were examined, weighed and their litter changed daily, respecting the
106 guide of good practice and animal welfare.
107
108 OFA rats were anesthetized by intravenous perfusion of ketamine (50mg/kg) and
109 Acepromazine (0.5mg/kg). Anesthesia for wistar rats was induced and maintained by
110 inhalation of an O2-Isoflurane mixture (IsoFlo). Before surgery, OFA and wistar female
111 rats were weighed and injected with buprenorphine (Buprecare at 0.05 mg/kg) and
112 meloxicam (Meloxidolor at 0.5 mg/kg) subcutaneously. After anesthesia, animals were
113 shaved in the abdominal region, disinfected with polyviode iodine and wiped with 70%
114 isopropyl alcohol then a sterile drape placed at the operative site after. One ml
115 subcutaneous Lidocaine 0.1%. was injected prior to the cutaneous incision. An abdominal
116 vertical skin incision was performed followed by dissection with scissors of the
117 subcutaneous and muscle fascia tissues. The peritoneum was opened with scissors,
118 allowing access to the abdominal cavity to rule-out organic disease of the reproductive
119 system (uterus, oviducts, and ovaries). The uterus was exteriorized for the procedure. For
120 both types of animals, Buprenorphine (0.02mg/kg) was systemically administered twice a
121 day for 3 days. At the end of each protocol or in case of reaching the tolerance limit
122 point, animals were euthanized by lethal injection of sodium pentobarbital after
123 anesthesia by ketamine to limit the pain. No animals were reused for other experiments.
124
125 ***In vivo evaluation of the impact of DPF on endometrial thickness***

The objective was to evaluate the impact of DPF on the rat's uterine horn endometrial thickness as measured histologically. This study was performed on seven OFA female rats. After animal preparation, the right horn's extremity was opened and DPF inserted (Supplementary figure S1A). No DPF was placed in the left horn (control). The horns' extremities were ligated to limit film expulsion (Supplementary figure S1B). Operative and post-operative complications were recorded. Histological analysis was performed after euthanasia and salpingectomy at Day 1 (2 rats), Day 5 (3 rats) and Day 12 (2 rats). The last time point coincides with the expected full degradation of DPF in rats. Horns were split longitudinally, fixed in formalin (formaldehyde 3.5% and phosphate buffered saline) and embedded in paraffin wax. Samples were analyzed after Hematoxylin-Eosin-Safran (HES) staining. The endometrial thickness was measured microscopically in three sections, at the center of the horn and each of the extremities, using NDP view 2, Hamamatsu® software, and the average endometrial thickness calculated for each horn (Supplementary figure S2).

In vivo evaluation of the impact of DPF on fertility

Reproductive outcome was evaluated in a prospective randomized study on 20 OFA female rats. Randomization was used to determine which horn to be implanted. The selected horn was incised, DPF was inserted and the horn was sutured while the other horn was used as control. The main endpoint was the number of pregnancies per horn. The purpose of the test was to demonstrate the non-inferiority of the DPF treated horns compared to controls. Female rats were monitored in individual cages for 15 days to allow for abdominal healing and DPF degradation as demonstrated in our previous study [16]. On Day 15, they were mated with 5 OFA males to mitigate potential bias caused by variation in sperm parameters. Fifteen days after matting, manual palpation of the

abdomen and abdominal ultrasound were used to confirm pregnancies. Animals were then euthanized and the number of fetuses counted in each horn.

In vivo evaluation of the biological response to DPF

In total, 40 wistar female rats contributed to this experiment. The animals were randomly allocated to one of six groups arranged in 3 pairs: Groups 1 and 2 (5 intervention and 5 controls sampled 24 hours after surgery); Groups 3 and 4 (5 intervention and 5 controls sampled 5 days after surgery and Groups 5 and 6 (10 intervention with left horn implantation and 10 controls with left horn incision only, sampled 28 days after surgery). A 4 cm longitudinal midline abdominal incision from the urinary bladder was made. The uterine horn was exteriorized from the abdominal cavity ligated close to the uterine junction with the other uterine horn without cutting off blood circulation. An incision was made and either 3mg DPF introduced (intervention) or a sham incision without DPF implantation (control) depending on the rat group allocation. The incision was then sutured and the uterine horn replaced in the abdominal cavity. Depending on the group allocation, the same procedure was repeated on the second uterine horn if required.

Based on the *a priori* sampling time, the animal was euthanized and the uterine horns and the draining inguinal lymph nodes were preserved in a formalin - acetic acid – alcohol (AFA) medium, dehydrated then embedded in paraffin wax. Sections of about 4µm were stained with haematoxylin-eosin (H&E) before assessment using the NF-ISO 1993-6 grading system (available upon request). The irritation score was calculated based on the mean of the sum of tissue damage (necrosis) and cellular inflammatory parameter scores weighted with a factor of 2 plus the repair phase of inflammation (fibrosis and

neovascularization) and fatty infiltrate parameter scores. The irritation index reflecting the intensity of the inflammatory process and the local tissue effects was determined by subtracting the irritation score of control from the score of DPF implanted animals within each of the paired groups. An irritation index of 0 – 2.9, 3.0 – 8.9, 9.0 – 15.0 and >15 was considered non-, slightly, moderately, or severely irritant respectively. Distant organs (e.g stomach, intestine, rectum, liver, spleen, inguinal nodes, heart, aorta, pancreas, kidneys and vagina) were macroscopically and microscopically evaluated at 28 days.

In vivo evaluation of DPF efficacy on IUA prevention

In total, there were 24 animals divided into 3 groups of 8 rats each: Group 1 (15 horns abraded and treated with DPF + one control horn); Group 2 (15 horns abraded and treated with hyaluronic acid (HA) gel [0.5 ml, Hyalobarrier, Nordic Pharma, France] + one control horn; Group 3 – sham group - (15 horns abraded but not treated and one control horn). The 3 control horns did not undergo the abrasion procedure and did not receive any treatment and acted as histological references for a healthy horn. From the urinary bladder level a 4 cm longitudinal midline abdominal incision was made. The uterine horn was exteriorized from the abdominal cavity and incised over its entire length. Depending on group allocation, the endometrial lining was abraded by scratching with a scalpel blade to remove the epithelial layer + / - implanted [17]**.** The uterine horn was then ligated close to the uterine junction with the other horn and 2-3 mm under the abraded area without cutting off blood circulation, to keep the DPF and HA gel in contact with the uterine horn. After 7 days, the animals were euthanized and the uterine horns preserved in AFA, dehydrated then embedded in paraffin wax. Caudal, medial and cervical sections were taken from each horn and stained with H&E. The presence of intrauterine adhesions

was evaluated for each uterine horn. The incidence of IUA was defined as the presence of complete adhesion in at least one of the three sections of horn.

Statistical analysis

Prior to the reproduction study, a power analysis was conducted with a significance level of 5% based on the variance from a statistical two sample t-test resulted in a power of 80%. The mean endometrial thickness, calculated from 3 measurements per horn, were compared in the intervention and control arms using the Mann-Whitney U test. The mean number of pregnancies per horn were compared in the intervention and control arms using a paired student's t-test. Fertility non-inferiority was based on the difference in the average number of ongoing and prematurely terminated pregnancies in the DPF and control groups. The *a priori* thresholds for non-inferiority were -1 and +0.5 for the total number of ongoing pregnancies and the number of prematurely terminated pregnancies respectively. The lower and upper bounds of the confidence intervals for the difference in the average number of ongoing and terminated pregnancies respectively were compared to these thresholds to assess non-inferiority. The efficacy of DPF for IUA prevention was assessed using Fisher's exact test because the comparison of proportions of the existence of adhesions was on a small sample of < 30.

RESULTS

In vivo evaluation of the impact of DPF on endometrial thickness

The endometrial thickness was noted to be thinner on day one in the DPF compared to the control group. However the difference in thickness at all the implantation time periods (one, 5 and 12 days) was not significantly different between groups (Table 1).

In vivo evaluation of the impact of DPF on fertility

A total of 144 and 131 pregnancies were clinically observed in the DPF and control groups respectively and this difference was not statistically significant. The average of total pregnancies identified per horn was 7.2 (+/- 2.56) for the DPF group compared to 6.5 (+/- 2.81) in the controls, a difference of 0.65, p=0.38. While, there were 9 prematurely terminated pregnancies in the polymer group and 18 in the control group (p = 0.09). The mean difference in ongoing pregnancy between DPF and control groups was 1.1 (95% CI [0.089; + inf]). Whereas the mean difference in prematurely terminated pregnancies was - 0.45 (95%CI [- inf; -0.007]). These results support DPF non-inferiority because the lower bound of the confidence interval for the ongoing pregnancy mean difference in ongoing pregnancy was greater than, and the upper bound of the confidence interval for the mean difference in prematurely terminated pregnancies was lower than the pre-set non inferiority threshold of -1 and + 0.5 respectively.

In vivo evaluation of the biological response to DPF

One animal in the control group was found dead on day 7 and missing small intestines and caecum were the only macroscopic findings recorded at necropsy. Apart from this, we did not observe any unexpected clinical signs in either the DPF or control groups. Macroscopic and microscopic examination of the animals at 28 days did not reveal changes in the main organs of the treated or control groups. There were no macroscopic anomalies seen in the inguinal lymph nodes draining implantation sites. Figure 1 illustrates an example of the histopathological examinations of uterine horns at 24 hours, 5 days and 28 days after implantation in both the DPF and control groups. Examinations showed that 24 hours after implantation, DPF induced a minimal to mild inflammatory process within the endometrium in 4 out of 5 animals. This was characterized by an

infiltration of polymorphonuclear cells and/or macrophages and was associated with flattening of the epithelial cells, focal or localized extensive erosion and a luminal exudate in the DPF group when compared to control uterine horns. At five days, the induced inflammatory process was still observed within the endometrium but at a lower incidence (3/5 animals) and severity when compared to the 24-hour period. At 28 days, the macroscopic examination of the implantation sites showed that the segment of uterine horns between the ligature made during the implantation procedure and the ovaries were hypertrophied and fluid filled in 8/10 and 8/9 females from the DPF and control groups respectively. These changes were bilateral in 7 of the 8 affected animals in each group. Histopathological examinations showed that luminal dilatation, recorded in 5/10 and 8/9 females of the DPF and control group, respectively, was the histological correlate of the hypertrophied uterine horns observed at necropsy. Hence, these findings were deemed secondary to the implantation procedure. No mortality attributable to DPF occurred during the study. Table 2 displays the biological response of uterine tissues in DPF-treated and control groups. The DPF was classified as slightly irritant (irritation index 3.6) at the end of the 24-hour period and non-irritant at the end of the 5-day period (irritation index1.6) and 28-day period (irritation index 0) (raw data available on request).

In vivo evaluation of DPF efficacy on IUA prevention

In the sham group, 100% (15/15 horns) had at least one complete IUA (Figure 2A) among the 3 regions of the uterine horn. When comparing the DPF and HA treated groups, 26.7% (4/15) and 80% (12/15) horns had at least one complete adhesion respectively (p=0.009; 66% reduction) (Figure 2).

Discussion

Summary of findings

Our study showed that, although the endometrium was thinner on day one in the DPF implanted animals, it did not have a significant impact on endometrial thickness at any of the tested time periods or on pregnancy related outcomes compared to the control group. We also demonstrated that DPF was biologically well tolerated with no evidence that it induces any signs of tissue toxicity. Although DPF was classified as slightly irritant 24 hours after insertion, this irritation seemed to be short-lived because it did not persist at later assessments. Finally, DPF significantly reduced the likelihood of complete IUA formation compared to HA gel and sham-implanted animals, where 27% only of the animals had at least one complete adhesion compared to 80% and 100% respectively. The presence of IUA in the DPF groups could be due to faster polymer degradation in some rats. Some areas of horns not covered by the DPF due to fragmentation may therefore be more susceptible to adhesion formation. In the case of HA gel, and due to the difference in galenic form (gel vs film), the areas susceptible to adhesion formation would be more numerous, which could explain the higher rate of adhesions in the HA gel group., This observation supports the efficacy of DPF in preventing IUA formation after intrauterine procedures and concurs with previous reports demonstrating its efficacy in preventing intra-peritoneal adhesions [15,16].

Findings in light of what is known

IUA are an important cause of infertility, implantation failure, miscarriage and obstetric complications [3]. The exact mechanism of their formation is unknown and several management modalities have been proposed for their prevention and treatment [14]. Although there are several studies evaluating the impact of IUA on different clinical outcomes, there is paucity of information in relation to the efficacy of such interventions

on improving fertility [13]. Indeed, although guidelines acknowledge that the use of solid (IUD, balloon) or semi solid (gel) barrier methods reduce postoperative IUA reformation, the use of such barriers is not explicitly recommended, probably due to the lack of fertility data to justify their recommendation [18]. Furthermore, available barrier agents for the prevention of intraperitoneal adhesions cannot be used in the uterine cavity via the vaginal route [13,19]. It is also important to recognize that adhesions can form anywhere within the uterine cavity, hence, it is imperative that a device intended to reduce such adhesions should cover the entire uterine cavity. Indeed, in this study we were able to confirm DPF's efficacy in preventing IUA at multiple uterine sections within the same horn. Furthermore, our study is quite unique in that it also addressed the local and distant tissue response to the polymer film, which is essential information for the development and licensing of such a medical device. Therefore, we believe that the findings of our study demonstrating the efficacy of DPF in preventing IUA, its non-inferiority in relation to fertility outcomes and its minimal biological effect on the endometrium have a significant clinical potential.

Strengths, limitations and future implications

We appreciate that our study has some limitations. First, our findings are based on an animal study. Nonetheless, rats are a good model for the evaluation of IUAs and are largely used in similar experimental studies because of the relative ease in managing their reproduction. Furthermore, their bicornuate uterus enables the animal to act as its own control [20]. Therefore, our choice of this model was built on currently available data of similar study designs because demonstrating DPF efficacy in a model with high tendency for IUA formation will likely be effective in a lower risk situation such as post miscarriage curettage [17,20]. Second, in addition to assessing the macroscopic and

microscopic effects of DPF on the endometrium and draining lymph nodes, the evaluation of inflammatory markers would have complemented our results [21]. However, the ability to test the impact of DPF on endometrial thickness, fertility, biological responses on surrounding tissues and IUA prevention in one study is a major strength to our work. The natural progression now is to validate our results in humans to assess the efficacy of DPF on IUA prevention in women. Some adjustments were performed to adapt the polymeric film to the shape and dimensions of the human uterus and to facilitate its trans-cervical insertion into the uterine cavity while mitigating the risk of any concomitant tissue trauma. This is currently under clinical investigation after hysteroscopic myomectomy to assess DPF efficacy as a preventive barrier against IUA (NCT04381728).

Conclusions

In conclusion, DPF has optimal degradation properties, no long-term tissue irritation and no negative impact on fertility outcomes in rats. Moreover, DPF significantly reduced the risk of IUA formation in the tested animals compared to HA gel or no treatment. Therefore, DPF seems to be a potentially promising innovation for the prevention of intrauterine synechiae and their recurrence.

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Authors' contribution:

SH: Planning of the project, protocol testing and its implementation, animal surgical preparation and testing, analysis of the data, writing and editing of the article.

SL: Planning of the project, test protocols writing, implementation and supervising analysis of the data, writing and editing of the article.

LA: Animal surgical preparation and testing

SW: Animal surgical preparation and testing , participated in the drafting of the introduction

IL: Animal surgical preparation and testing

HT: animal surgical preparation and testing

374 XG: planning of the project, analysis of the data, writing and editing of the article

375 RdT: planning of the project, participated of the drafting of the article

376 VL: planning of the project, participated of the drafting of the article

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Captions:

Tables

- Table 1: Endometrial thickness measurements (µm) in the DPF group and control group, at Day 1, Day 5 and Day 12.
- Table 2: Irritation scores of uterine tissues in DPF and control groups at each interval time (24 hours, 5 days and 28 days) and calculated irritation index of DPF.

Figures

- Figure 1: Histological examination (HES staining) of the uterine horns: (A,B,C) DPF group at 1 day, 5 days and 28 days respectively (D,E,F) sham group at 1 day, 5 days and 28 days respectively.
- Figure 2: Prevention of intrauterine adhesion. (A-D) Histology: HES staining of uterine horns sections at 7 days (A) Sham group: the horn is fully closed by complete IUA (B) HA group: the horn is fully closed by complete IUA (C) DPF group: the lumen is visible, no IUA (D) Control group: the lumen is visible, no IUA. (E) Number of uterine horns in percent (%) presenting at least one complete IUA at day 7 following uterine curettage in the sham group, HA group and DPF group.

Fig 1

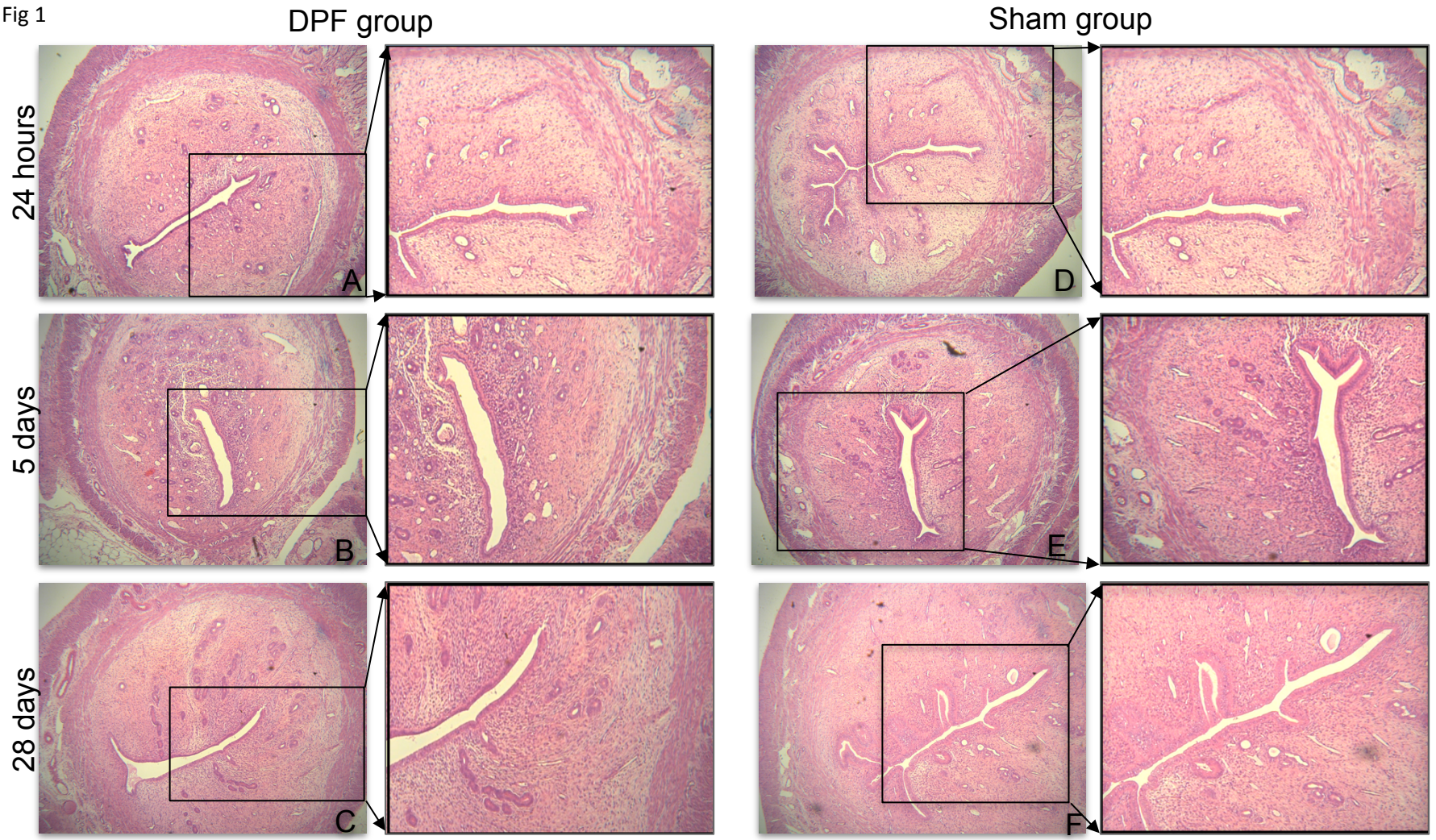
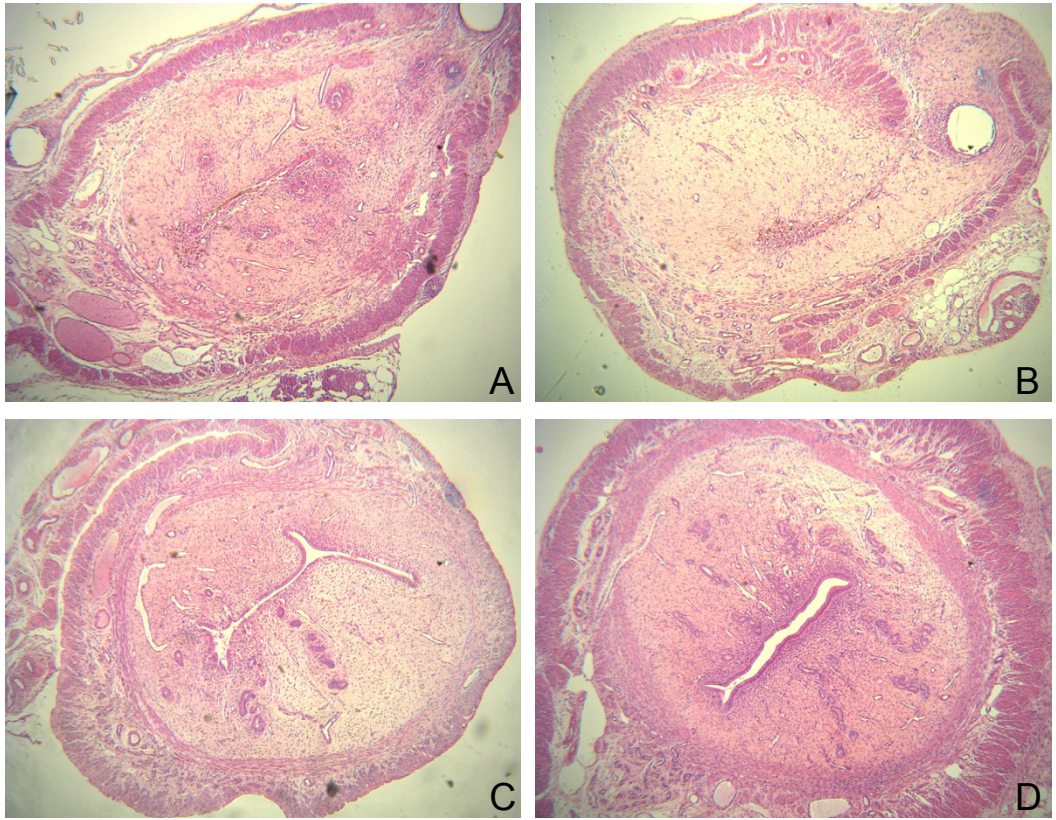
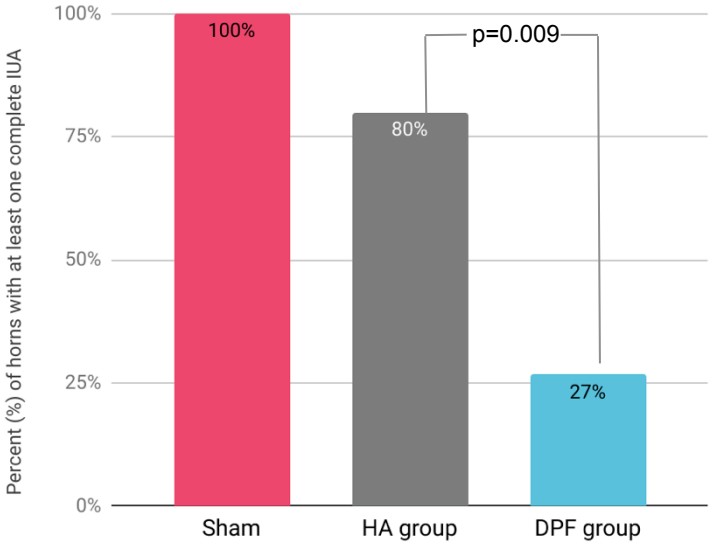


Fig 2


A
B
C
D


Percent (%) of horns with at least one complete IUA
100%
75%
50%
25%
0%
100%
80%
27%
p=0.009
Sham
HA group
DPF group

	DPF group (n=7)	Control group (n=7)	p
Day 1	337.3 +/-118.9	693 +/- 371.8	0.09
Day 5	897+/-441	927.8 +/- 226	1
Day 12	647.3+/- 147.9	777 +/- 406.5	0.2

Table 1: Endometrial thickness measurements (µm) in the DPF group and control group, at Day 1, Day 5 and Day 12

Table 2: Mean irritation scores of uterine tissues in DPF and control groups at each interval time (24 hours, 5 days and 28 days) and calculated irritation index of DPF.

Time-period	Group	Mean Irritation Score	DPF Irritation Index
24-hour	Treated with DPF	3.6 +/- 2.95	3.6 (Slightly irritant)
	Sham group	0.0 +/- 0.0	
5-day	Treated with DPF	1.6 +/-1.57	1.6 (Non irritant)
	Sham group	0.0 +/- 0.0	
28-day	Treated with DPF	0.0 +/- 0.0	0.0 (Non irritant)
	Sham group	0.0 +/- 0.0	