

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

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## 2 film for the prevention of intrauterine adhesions

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- start-up company based on the results of the current study, and one of the authors (S.L.)

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

## 28 Abstract

29 *Objective:* To study the safety of DPF (degradable polymeric film) and its efficacy on

30 reducing the risk of intrauterine adhesions (IUA) formation in a rat model.

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

32 *Setting:* University and good practice animal laboratories.

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

35 *Intervention(s) and measurements:* OFA rats were used for in vivo evaluation of the

36 impact of DPF on endometrial thickness and in vivo evaluation of the effect on fertility.

37 For in vivo evaluation of the biological response, 40 wistar rats were randomly allocated

38 to intervention and control groups with matched sampling time after surgery. Finally, for

39 the in vivo evaluation of DPF efficacy on IUA prevention, a total of 24 wistar rats were

40 divided into 3 groups that were treated with DPF, hyaluronic acid (HA) gel and a sham

41 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

45 days. Finally, DPF significantly reduced the likelihood of complete IUA formation

46 compared to HA gel and sham implanted animals, where only 27% of the animals had

47 their uterine cavity obliterated compared to 80% and 100% respectively.

48 *Conclusion:* DPF is a safe degradable polymeric film that is effective in preventing IUA

49 formation after intrauterine curettage in rats.

50

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

#### 52 Introduction

53 Intrauterine adhesions (IUA) result from an endometrial trauma leading to a partial or 54 complete obliteration of the uterine cavity [1,2]. A Meta-analysis of 10 RCTs involving 55 912 women calculated an IUA prevalence following miscarriage of 19.1% (95% CI 12.8-56 27.5%) [3]. IUA can also occur after intrauterine surgical interventions for the 57 management of uterine leiomyomas, septa and polyps. Diagnosis can be suspected when 58 pain or abnormal bleeding occur after surgical or obstetrical intrauterine trauma [4]. 59 Although the iatrogenic trauma is established as one of the main precipitating factors, the 60 exact underlying pathophysiology of IUA formation is less well known. IUA can lead to 61 primary or secondary subfertility, early or late miscarriage and increase the risk for 62 several obstetric complications including abnormal placentation, postpartum 63 hysterectomy, prematurity and cesarean section [3,5–8] 64 65 Hysteroscopy remains the mainstay for IUA diagnosis and treatment, nevertheless, carry 66 the risk of IUA recurrence [9–11]. Several anti-adhesive strategies including hormonal 67 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 68 69 the incidence and severity of de novo IUA after operative hysteroscopy [13], there is 70 limited evidence relating to their effectiveness on improving pregnancy rates [14]. We 71 previously described the efficacy of a degradable polymeric film (DPF), in a rat model, 72 on post-operative abdominal adhesion [15,16]. The aim of the present study was to assess 73 the safety of DPF, its efficacy on reducing the risk of IUA formation and impact on 74 fertility in a rat model.

75

#### 76 Material and methods

#### 77 Polymer synthesis and shaping

78 DPF was synthesized at the biopolymer laboratory of Montpellier, France, and previously 79 described by Leprince et al. [16]. For the evaluation of endometrial thickness and fertility 80 impact, DPF was shaped by hot extrusion with a Noztek Powder Filament Extruder 81 (Shoreham, England) to obtain a polymer filament with a diameter of 1.5 mm. For the 82 evaluation of biological response and the evaluation of IUA prevention, DPF was 83 synthesized at Polymer Expert (Pessac, France) and shaped by hot-pressure to obtain a 84 film (10 mg; length 1.3 +/-0.1 cm). The co-polymer was sterilized by irradiation between 85 27.7 and 36.6 kGy by Ionisos (Chaumesnil, France). DPF is now manufactured under the 86 brand name of "Womed Leaf".

87

#### 88 Animal preparation

89 The evaluation of endometrial thickness and fertility were conducted at the Experimental 90 Department of University of Montpellier (Montpellier, France) on OFA (Oncins France 91 Strain A) female rats (7-8 weeks old) weighing between 250 and 275 grams. OFA male 92 rats weighing between 275 and 300 grams (purchased from Earl Cegav SSC, St Mars 93 d'Egrenne, France) were also required for the fertility experiment. While the evaluation 94 of biological response and the evaluation of IUA prevention were conducted at Phycher 95 Bio Development (Pessac, France), under good laboratory practice, on female wistar rats 96 (SPF Caw; 9-10 weeks old), supplied by Janvier Labs. The animal strain used depended 97 on availability at the time of conducting the experiment. All animal investigations were 98 approved by the Ethics Committee of the French Ministry of Education and Research 99 (contract number 02367.01, task order 1065 and EC N°.76) and carried out in accordance 100 with the EU Directive 2010/63/EU for animal experiments. All efforts were made to 101 minimize animal suffering or distress, and to use the minimum number of animals

necessary to produce reliable scientific data. All the animals were in quarantine for one
week prior to treatment. They were placed individually in an air-conditioned animal
holding facility (22°C +/- 3°C; 30%-70% humidity) with free access to food (SAFE®)
and water. They were examined, weighed and their litter changed daily, respecting the
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

126 The objective was to evaluate the impact of DPF on the rat's uterine horn endometrial 127 thickness as measured histologically. This study was performed on seven OFA female 128 rats. After animal preparation, the right horn's extremity was opened and DPF inserted 129 (Supplementary figure S1A). No DPF was placed in the left horn (control). The horns' 130 extremities were ligated to limit film expulsion (Supplementary figure S1B). Operative 131 and post-operative complications were recorded. Histological analysis was performed 132 after euthanasia and salpingectomy at Day 1 (2 rats), Day 5 (3 rats) and Day 12 (2 rats). 133 The last time point coincides with the expected full degradation of DPF in rats. Horns 134 were split longitudinally, fixed in formalin (formaldehyde 3.5% and phosphate buffered 135 saline) and embedded in paraffin wax. Samples were analyzed after Hematoxylin-Eosin-136 Safran (HES) staining. The endometrial thickness was measured microscopically in three 137 sections, at the center of the horn and each of the extremities, using NDP view 2, 138 Hamamatsu® software, and the average endometrial thickness calculated for each horn 139 (Supplementary figure S2).

140

## 141 In vivo evaluation of the impact of DPF on fertility

142 Reproductive outcome was evaluated in a prospective randomized study on 20 OFA 143 female rats. Randomization was used to determine which horn to be implanted. The 144 selected horn was incised, DPF was inserted and the horn was sutured while the other 145 horn was used as control. The main endpoint was the number of pregnancies per horn. 146 The purpose of the test was to demonstrate the non-inferiority of the DPF treated horns 147 compared to controls. Female rats were monitored in individual cages for 15 days to 148 allow for abdominal healing and DPF degradation as demonstrated in our previous study 149 [16]. On Day 15, they were mated with 5 OFA males to mitigate potential bias caused by 150 variation in sperm parameters. Fifteen days after matting, manual palpation of the

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

153

### 154 In vivo evaluation of the biological response to DPF

155 In total, 40 wistar female rats contributed to this experiment. The animals were randomly 156 allocated to one of six groups arranged in 3 pairs: Groups 1 and 2 (5 intervention and 5 157 controls sampled 24 hours after surgery); Groups 3 and 4 (5 intervention and 5 controls 158 sampled 5 days after surgery and Groups 5 and 6 (10 intervention with left horn 159 implantation and 10 controls with left horn incision only, sampled 28 days after surgery). 160 A 4 cm longitudinal midline abdominal incision from the urinary bladder was made. The 161 uterine horn was exteriorized from the abdominal cavity ligated close to the uterine 162 junction with the other uterine horn without cutting off blood circulation. An incision was 163 made and either 3mg DPF introduced (intervention) or a sham incision without DPF 164 implantation (control) depending on the rat group allocation. The incision was then 165 sutured and the uterine horn replaced in the abdominal cavity. Depending on the group 166 allocation, the same procedure was repeated on the second uterine horn if required.

167

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 175neovascularization) and fatty infiltrate parameter scores. The irritation index reflecting176the intensity of the inflammatory process and the local tissue effects was determined by177subtracting the irritation score of control from the score of DPF implanted animals within178each of the paired groups. An irritation index of 0 - 2.9, 3.0 - 8.9, 9.0 - 15.0 and >15179was considered non-, slightly, moderately, or severely irritant respectively. Distant organs180(e.g stomach, intestine, rectum, liver, spleen, inguinal nodes, heart, aorta, pancreas,181kidneys and vagina) were macroscopically and microscopically evaluated at 28 days.

182

#### 183 In vivo evaluation of DPF efficacy on IUA prevention

184 In total, there were 24 animals divided into 3 groups of 8 rats each: Group 1 (15 horns 185 abraded and treated with DPF + one control horn); Group 2 (15 horns abraded and treated 186 with hyaluronic acid (HA) gel [0.5 ml, Hyalobarrier, Nordic Pharma, France] + one 187 control horn; Group 3 – sham group - (15 horns abraded but not treated and one control 188 horn). The 3 control horns did not undergo the abrasion procedure and did not receive 189 any treatment and acted as histological references for a healthy horn. From the urinary 190 bladder level a 4 cm longitudinal midline abdominal incision was made. The uterine horn 191 was exteriorized from the abdominal cavity and incised over its entire length. Depending 192 on group allocation, the endometrial lining was abraded by scratching with a scalpel 193 blade to remove the epithelial layer + / - implanted [17]. The uterine horn was then 194 ligated close to the uterine junction with the other horn and 2-3 mm under the abraded 195 area without cutting off blood circulation, to keep the DPF and HA gel in contact with the 196 uterine horn. After 7 days, the animals were euthanized and the uterine horns preserved in 197 AFA, dehydrated then embedded in paraffin wax. Caudal, medial and cervical sections 198 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 ofcomplete adhesion in at least one of the three sections of horn.

201

#### 202 Statistical analysis

203 Prior to the reproduction study, a power analysis was conducted with a significance level 204 of 5% based on the variance from a statistical two sample t-test resulted in a power of 205 80%. The mean endometrial thickness, calculated from 3 measurements per horn, were 206 compared in the intervention and control arms using the Mann-Whitney U test. The mean 207 number of pregnancies per horn were compared in the intervention and control arms 208 using a paired student's t-test. Fertility non-inferiority was based on the difference in the 209 average number of ongoing and prematurely terminated pregnancies in the DPF and 210 control groups. The *a priori* thresholds for non-inferiority were -1 and +0.5 for the total 211 number of ongoing pregnancies and the number of prematurely terminated pregnancies 212 respectively. The lower and upper bounds of the confidence intervals for the difference in 213 the average number of ongoing and terminated pregnancies respectively were compared 214 to these thresholds to assess non-inferiority. The efficacy of DPF for IUA prevention was 215 assessed using Fisher's exact test because the comparison of proportions of the existence 216 of adhesions was on a small sample of < 30.

217

#### 218 **RESULTS**

219 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 tothe control group. However the difference in thickness at all the implantation time

222 periods (one, 5 and 12 days) was not significantly different between groups (Table 1).

#### 224 In vivo evaluation of the impact of DPF on fertility

225 A total of 144 and 131 pregnancies were clinically observed in the DPF and control 226 groups respectively and this difference was not statistically significant. The average of 227 total pregnancies identified per horn was 7.2 (+/- 2.56) for the DPF group compared to 228 6.5 (+/-2.81) in the controls, a difference of 0.65, p=0.38. While, there were 9 229 prematurely terminated pregnancies in the polymer group and 18 in the control group (p 230 = 0.09). The mean difference in ongoing pregnancy between DPF and control groups was 231 1.1 (95% CI [0.089; + inf]). Whereas the mean difference in prematurely terminated 232 pregnancies was - 0.45 (95%CI [- inf; -0.007]). These results support DPF non-inferiority 233 because the lower bound of the confidence interval for the ongoing pregnancy mean 234 difference in ongoing pregnancy was greater than, and the upper bound of the confidence 235 interval for the mean difference in prematurely terminated pregnancies was lower than 236 the pre-set non inferiority threshold of -1 and +0.5 respectively.

237

#### 238 In vivo evaluation of the biological response to DPF

239 One animal in the control group was found dead on day 7 and missing small intestines 240 and caecum were the only macroscopic findings recorded at necropsy. Apart from this, 241 we did not observe any unexpected clinical signs in either the DPF or control groups. 242 Macroscopic and microscopic examination of the animals at 28 days did not reveal 243 changes in the main organs of the treated or control groups. There were no macroscopic 244 anomalies seen in the inguinal lymph nodes draining implantation sites. Figure 1 245 illustrates an example of the histopathological examinations of uterine horns at 24 hours, 246 5 days and 28 days after implantation in both the DPF and control groups. Examinations 247 showed that 24 hours after implantation, DPF induced a minimal to mild inflammatory 248 process within the endometrium in 4 out of 5 animals. This was characterized by an

249 infiltration of polymorphonuclear cells and/or macrophages and was associated with 250 flattening of the epithelial cells, focal or localized extensive erosion and a luminal 251 exudate in the DPF group when compared to control uterine horns. At five days, the 252 induced inflammatory process was still observed within the endometrium but at a lower 253 incidence (3/5 animals) and severity when compared to the 24-hour period. At 28 days, 254 the macroscopic examination of the implantation sites showed that the segment of uterine 255 horns between the ligature made during the implantation procedure and the ovaries were 256 hypertrophied and fluid filled in 8/10 and 8/9 females from the DPF and control groups 257 respectively. These changes were bilateral in 7 of the 8 affected animals in each group. 258 Histopathological examinations showed that luminal dilatation, recorded in 5/10 and 8/9 259 females of the DPF and control group, respectively, was the histological correlate of the 260 hypertrophied uterine horns observed at necropsy. Hence, these findings were deemed 261 secondary to the implantation procedure. No mortality attributable to DPF occurred 262 during the study. Table 2 displays the biological response of uterine tissues in DPF-263 treated and control groups. The DPF was classified as slightly irritant (irritation index 264 3.6) at the end of the 24-hour period and non-irritant at the end of the 5-day period 265 (irritation index 1.6) and 28-day period (irritation index 0) (raw data available on request). 266

## 267 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).

272

## 273 **Discussion**

### 274 Summary of findings

275 Our study showed that, although the endometrium was thinner on day one in the DPF 276 implanted animals, it did not have a significant impact on endometrial thickness at any of 277 the tested time periods or on pregnancy related outcomes compared to the control group. 278 We also demonstrated that DPF was biologically well tolerated with no evidence that it 279 induces any signs of tissue toxicity. Although DPF was classified as slightly irritant 24 280 hours after insertion, this irritation seemed to be short-lived because it did not persist at 281 later assessments. Finally, DPF significantly reduced the likelihood of complete IUA 282 formation compared to HA gel and sham-implanted animals, where 27% only of the 283 animals had at least one complete adhesion compared to 80% and 100% respectively. The 284 presence of IUA in the DPF groups could be due to faster polymer degradation in some 285 rats. Some areas of horns not covered by the DPF due to fragmentation may therefore be 286 more susceptible to adhesion formation. In the case of HA gel, and due to the difference 287 in galenic form (gel vs film), the areas susceptible to adhesion formation would be more 288 numerous, which could explain the higher rate of adhesions in the HA gel group., This 289 observation supports the efficacy of DPF in preventing IUA formation after intrauterine 290 procedures and concurs with previous reports demonstrating its efficacy in preventing 291 intra-peritoneal adhesions [15,16].

292

#### 293 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

299 on improving fertility [13]. Indeed, although guidelines acknowledge that the use of solid 300 (IUD, balloon) or semi solid (gel) barrier methods reduce postoperative IUA reformation, 301 the use of such barriers is not explicitly recommended, probably due to the lack of 302 fertility data to justify their recommendation [18]. Furthermore, available barrier agents 303 for the prevention of intraperitoneal adhesions cannot be used in the uterine cavity via the 304 vaginal route [13,19]. It is also important to recognize that adhesions can form anywhere 305 within the uterine cavity, hence, it is imperative that a device intended to reduce such 306 adhesions should cover the entire uterine cavity. Indeed, in this study we were able to 307 confirm DPF's efficacy in preventing IUA at multiple uterine sections within the same 308 horn. Furthermore, our study is quite unique in that it also addressed the local and distant 309 tissue response to the polymer film, which is essential information for the development 310 and licensing of such a medical device. Therefore, we believe that the findings of our 311 study demonstrating the efficacy of DPF in preventing IUA, its non-inferiority in relation 312 to fertility outcomes and its minimal biological effect on the endometrium have a 313 significant clinical potential.

314

### 315 Strengths, limitations and future implications

316 We appreciate that our study has some limitations. First, our findings are based on an 317 animal study. Nonetheless, rats are a good model for the evaluation of IUAs and are 318 largely used in similar experimental studies because of the relative ease in managing their 319 reproduction. Furthermore, their bicornuate uterus enables the animal to act as its own 320 control [20]. Therefore, our choice of this model was built on currently available data of 321 similar study designs because demonstrating DPF efficacy in a model with high tendency 322 for IUA formation will likely be effective in a lower risk situation such as post 323 miscarriage curettage [17,20]. Second, in addition to assessing the macroscopic and

324 microscopic effects of DPF on the endometrium and draining lymph nodes, the 325 evaluation of inflammatory markers would have complemented our results [21]. 326 However, the ability to test the impact of DPF on endometrial thickness, fertility, 327 biological responses on surrounding tissues and IUA prevention in one study is a major 328 strength to our work. The natural progression now is to validate our results in humans to 329 assess the efficacy of DPF on IUA prevention in women. Some adjustments were 330 performed to adapt the polymeric film to the shape and dimensions of the human uterus 331 and to facilitate its trans-cervical insertion into the uterine cavity while mitigating the risk 332 of any concomitant tissue trauma. This is currently under clinical investigation after 333 hysteroscopic myomectomy to assess DPF efficacy as a preventive barrier against IUA 334 (NCT04381728).

335

#### 336 Conclusions

337 In conclusion, DPF has optimal degradation properties, no long-term tissue irritation and

338 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.

340 Therefore, DPF seems to be a potentially promising innovation for the prevention of

341 intrauterine synechiae and their recurrence.

342

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349

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363

### 364 Authors' contribution:

- 365 SH: Planning of the project, protocol testing and its implementation, animal surgical
- 366 preparation and testing, analysis of the data, writing and editing of the article.
- 367 SL: Planning of the project, test protocols writing, implementation and supervising
- analysis of the data, writing and editing of the article.
- 369 LA: Animal surgical preparation and testing
- 370 SW: Animal surgical preparation and testing, participated in the drafting of the
- 371 introduction
- 372 IL: Animal surgical preparation and testing
- 373 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

377

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452		

454 <b>Captions:</b>	
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## 455 Tables

456	•	Table 1: Endometrial thickness measurements $(\mu m)$ in the DPF group and control
457		group, at Day 1, Day 5 and Day 12.
458	٠	Table 2: Irritation scores of uterine tissues in DPF and control groups at each
459		interval time (24 hours, 5 days and 28 days) and calculated irritation index of
460		DPF.
461		
462	Figure	25
463	٠	Figure 1: Histological examination (HES staining) of the uterine horns: (A,B,C)
464		DPF group at 1 day, 5 days and 28 days respectively (D,E,F) sham group at 1 day,
465		5 days and 28 days respectively.
466	٠	Figure 2: Prevention of intrauterine adhesion. (A-D) Histology: HES staining of
467		uterine horns sections at 7 days (A) Sham group: the horn is fully closed by
468		complete IUA (B) HA group: the horn is fully closed by complete IUA ( C) DPF
469		group: the lumen is visible, no IUA (D) Control group: the lumen is visible, no
470		IUA. (E) Number of uterine horns in percent (%) presenting at least one complete
471		IUA at day 7 following uterine curettage in the sham group, HA group and DPF
472		group.
473		



Fig 2



	DPF group	Control group	
	(n=7)	(n=7)	р
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

Time-period	Group	Mean Irritation Score	DPF Irritation Index	
24-hour	Treated with DPF	3.6 +/- 2.95	3.6 (Slightly irritant)	
24 nour	Sham group	0.0 +/- 0.0	5.0 (Singhiry initialit)	
5-day	Treated with DPF	1.6 +/-1.57	1.6 (Non irritant)	
5 duy	Sham group	0.0 +/- 0.0		
28-day	Treated with DPF	0.0 +/- 0.0	0.0 (Non irritant)	
20 44	Sham group	0.0 +/- 0.0		

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.