

Histological Study on the Effect of Metronidazole (Flagyl®) on the Distribution of Collagen Fibers in the Uterus of the Pregnant *Rattus norvegicus* Raton Days 7 and 9 of Pregnancy
دراسة نسيجية (*) لآثار عقار Metronidazole (Flagyl®) على توزيع الياف الكولاجين في رحم اناث الجرذ النرويجي *Rattus norvegicus* في الايام السابع والتاسع من الحمل (*)

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Abstract

To determine the effect of Metronidazole (Flagyl®) on distribution of collagen fibers in the pregnant rat uterus during the implantation period on days 7 and 9 day post coitum (dpc), sixty-four female rats of confirmed pregnancy have been used ,divided into two treated groups (48 rats) received oral dosage commercial Metronidazole (Flagyl®) and one control group (16 rats) received distilled water via the same route. Histological technique was applied for sections taken from implantation sites at days 7 and 9 dpc. The sections were stained in Hematoxylin and Eosin and Gomori's one step-trichrome stains. The results indicates that blastocysts were able to implant successfully and have initiated the implantation reaction and decidualization in the uteri of all rats used. As a normal reaction on day 7 dpc collagen fibers was found to be virtually absent in the decidual tissue of primary decidual zone (PDZ) and secondary decidual zone (SDZ) in contrast to undifferentiated zone (UDZ) in which the fibers was widely distributed.

On day 9 dpc collagen fibers were abundant in the mesometrialdecidual zone (MDZ). Collagen fibers were an important support to the blood vessels in this area which represents a prime route for establishment of nutrient supply through maternal blood vessels supplying the chorioallantoic

placenta of rat. The results have revealed that oral intake of Metronidazole did not interfere with the normal process of implantation of rat blastocyst.

Key Words: Metronidazole, implantation, rat, decidualization, collagen fibers.

الخلاصة

لمعرفة تأثير عقار الميترونيدازول (فلاجيل®) (Flagyl®) التجاري على انتشار الكولاجين في رحم الجرذ الحامل خلال فترة الغرس في اليوم السابع والتاسع من بدء الحمل استعمل 64 أنثى جرذ تأكد حملهن في مجموعتين الاولى علاجية ضمت (48) جرذ تم تقسيمها إلى مجموعتين 24 لكل واحدة تم تجريعها العقار فمويا والثانية مجموعة سيطرة (16) جرذ انثى تم تجريعها ماء مقطر بنفس الطريقة. تم تطبيق التقنية النسيجية على مقاطع الرحم من أماكن الغرس في اليومين السابع والتاسع من الحمل ، حيث صبغت العينات بصيغة هيماتوكسيلينوايوسين وصيغة كوموري's Gomori's one step trichrome stains. اظهرت نتائج البحث يمكن الأكياس الأريمية Blastocysts من الانغراس وتكوين النسيج الساقط Decidualization في أرحام كل الجرذان. وكنتيجة طبيعية في اليوم السابع من الحمل (day 7 dpc) فان ألياف الكولاجين لم تكن موجودة في كل من مناطق الغشاء الساقط الأولى Primary Decidual Zone (PDZ) والثانوي Secondary Decidual Zone (SDZ) بينما كانت واسعة الانتشار في المنطقة غير المتميزة Undifferentiated Zone (UDZ). في اليوم التاسع (day 9 dpc) كثر تواجد الألياف الكولاجينية في منطقة الغشاء الساقط المسراق- رحمية Mesometrial Decidual Zone (MDZ). الألياف الكولاجينية كانت داعماً مهماً للأوعية الدموية في هذه المساحة التي تغذى اللقائقي المشيمي لسخذ الجرذ the chorioallantoic placenta. بينت هذه الدراسة أن تجريع عقار الميترونيدازول (Flagyl®) (Metronidazole) لم يتعارض مع العملية الطبيعية لغرس الكيسة الأريمية Blastocyst في الجرذان.

Introduction

Metronidazole (Flagyl®) is among many drugs which possessed direct trichomonacidal and amebacidal activity against *Trichomonas vaginalis* and *Entamebahistolytica* in females and males and it is active in vitro against most obligate anaerobes, in generally it is bactericidal [1;2;3;] Metronidazole are commonly used as bactericidal against anaerobic bacteria and is also active against *Giardia lamblia*[4;5;6;7]. Metronidazole has shown evidence of carcinogenic activity in a number of studies involving chronic, oral administration in mice and rats. Several long-term, oral-dosing studies in the rat have been completed[8;9]. There were statistically significant increases in the incidence of various neoplasms, particularly in mammary and hepatic tumors, among female rats administered metronidazole over those noted in the concurrent female control groups. Metronidazole crosses the placental barrier and enters the fetal circulation rapidly[10;11]. There are, however, no adequate and well-controlled studies in pregnant women[12]. Because animal reproduction studies are not always predictive of human response, and because Metronidazole is a carcinogen in rodents, this drug should be used during pregnancy only if clearly needed [13;14].

The question which has been repeatedly been asked: is the use of Metronidazole for varieties of clinical considerations, during pregnancy, associated with an increasing risk for interfering with implantation and subsequent success of pregnancy and a cause of congenital malformation or not?.

Normally the process of implantation is marked by development of decidual tissue [15;16;17]. As a consequences to implantation changes in the distribution of fibrillar components of the extracellular matrix have been noted in morphological studies of the rat uterus during pregnancy [18;19;20]. Aniline blue staining suggested the absence of collagen bundles throughout the decidual tissue[21]. On day 6-8 of pregnancy, decidual tissue was found to contain very little collagen when compared with non-implantation sites [22].

The aim of the present work was to assess the nature of changes in the distribution of collagen fibers in the uterus of the pregnant rat treated with Metronidazole and compare that with control group.

Materials and Methods

Sixty four, virgin female rats ,45 days old, weighting about 170-250 gms each were used in this study. They were maintained under light program of LD 12:12 and fed *ad libitum*. The animals were mated and the day on which spermatozoa were found in the vaginal smear and the presence of vaginal plug was designated day 1 of pregnancy.

The design of the experiment is outlined in table (1). All female rats were divided into two main groups: The G1 group was the rats sacrificed on day 7 of pregnancy (dpc), The G 2 group was the rats sacrificed on day 9 of pregnancy (dpc). Each group was subdivided into three subgroups in accordance to administration of drug, as in table 1.

Table 1: Showing the arrangement of experimental design.

Groups	Subgroups			
	Control Distilled water	Therap. Metro. 9mg	Double therap. Metro. 18mg	Triple therap. Metro. 27mg
G1 (7) dpc	n=8	n=8	n=8	n=8
G2 (9) dpc	n=8	n=8	n=8	n=8

G1 (7) = Groups of female rats that were scarified on day (7) dpc **G2 (9)** = Groups of female rats that were scarified on day (9) dpc **n**= Number of rats in each subgroups. **Control** = Control rats were dosages distilled water orally. **Therap. Metro.** = female rats were treated with 9mg (therapeutic) dose of the Metronidazole, **Double therap. Metro.** = female rats were treated with 18mg (double) therapeutic dose. **Triple therap. Metro.** = female rats were treated with 27mg (triple) therapeutic dose of the Metronidazole.

Administration of the drugs:

The Flagyl[®] was available as tablets, each one containing 500 mg of the active ingredient Metronidazole (From Sanofi Aventis Co. France).

Calculation of dose:

The dose for the rat was calculated according to Paget and Barnas^[23] equation: Metronidazole 9 mg/day was given for 200 gm body weight rat as therapeutic dose. The calculated dose (therapeutic, double and triple therapeutic dose) of suspension solution in distilled water of Metronidazole drugs were received oral dosage one time daily for six days (G1 group) to the animal sacrificed on the morning of day 7 dpc, and for eight days (G2 group) to the animals sacrificed on the morning of day 9 dpc. Distilled water was administered via the same route to the control animals of both groups G1 and G2.

Tissue sampling and processing:

After the animals were anesthetized, the uteri were dissected out and divided into segments containing the implantation sites. These sites were fixed in 10% formal saline for 48 hours. The samples then dehydrated in ascending grades of ethyl alcohol, cleared in xylene and embedded in paraffin wax. The blocks were carefully oriented to have the cross sections to be cut from the

implantation sites. Five μm thickness serial sections containing the implantation sites only were cut. The sections were deparaffinized and hydrated for Hematoxylin and Eosin staining (for general histological picture). For demonstrating the distribution of collagen fibers Gomori's rapid one step trichrome staining^[24] were followed. It stain up collagen fibers green.

Results

Day seven dpc (G1 group):

The findings for Hematoxylin and Eosin stained sections taken from the implantation sites of control and treated rats uterine at this day of pregnancy were showing the same results regarding the arrangement of decidual tissue. Similarly, the Gomori's one step trichrome stained sections have revealed no significant differences in the distribution of collagen fibers.

The initial site of endometrial stromal cells modification for decidualization which has been considered as an indication of successful implantation was in the antimesometrial side of endometrium. Subsequently decidualization has proceeded mesometrially. In the sections stained with Hematoxylin and Eosin four main zones could be identified (Fig. 1):



Figure 1- :Cross section in Rat uterus on 7 dpc (G1-Control. subgroup = female rats were orally dosage distilled water). Note the presence of decidual reaction. PDZ= Primary decidual zone; SDZ=secondary decidual zone; UBZ= undifferentiated basal zone, IZ = implantation zone; Ms= mesometrial side of endometrium; Am = antimesometrial side; My=myometrium. Hematoxylin and Eosin stain X20.

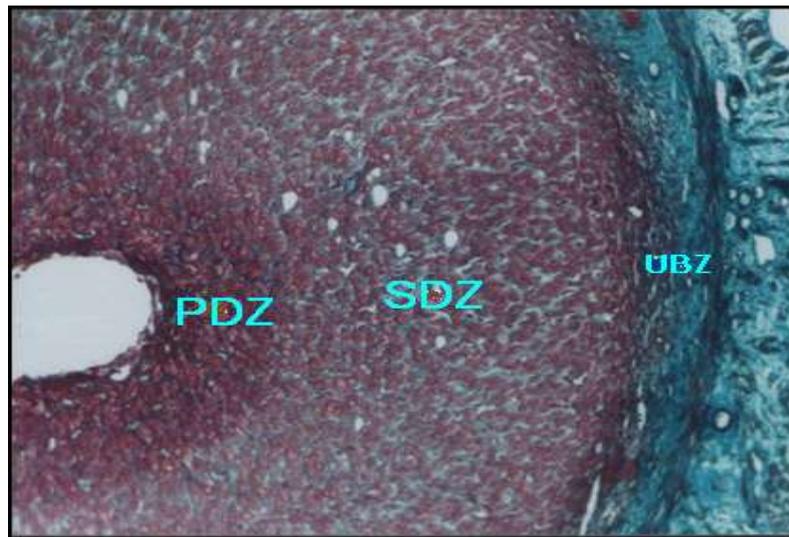


Figure 2 : Cross section in Rat uterus on day 7 dpc (subgroup Th. M.1 = female rats were treated with therapeutic dose of the Metronidazole). Note the accumulation of collagen fibers within the UBZ, moderate amount in SDZ, while no collagen fibers within PDZ. Gomori's one step trichrome stain. X40.

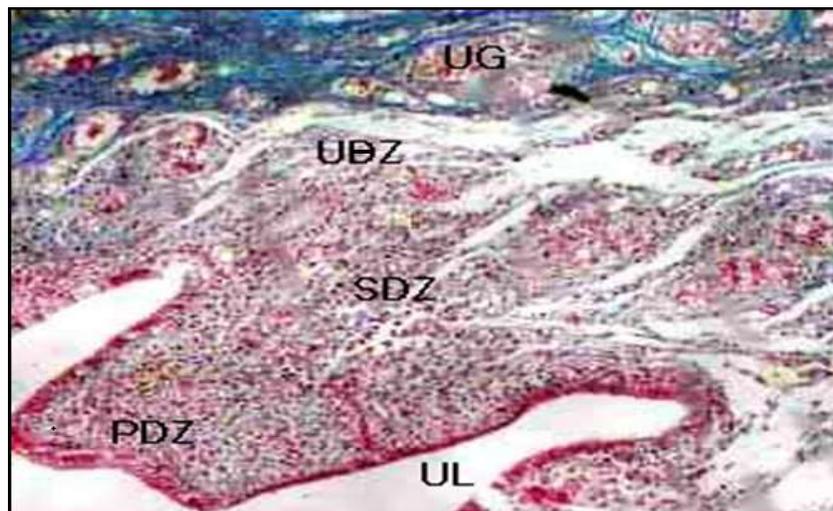


Figure 3 : Cross section in Rat uterus on day 7 dpc. (G1-Therap.Metro.subgroup, treated with double therapeutic dose of Metronidazole). Note the presence of decidual reaction. PDZ= Primary decidual zone; SDZ= secondary decidual zone; UBZ= undifferentiated basal zone; UL = Uterine Lumen; UG = Uterine Gland. Gomori's one step -trichrome stain, X100.

The primary decidual (PDZ): of closely packed decidual cells that surround the blastocyst and luminal epithelium. The secondary decidual zone (SDZ) situated between PDZ and Undifferentiated basal zone (UBZ) and occupying most of the area of endometrium forming a circle around the PDZ. The implantation zone (IZ) was a small zone located antimesometrial to the embryo where the epithelium was denuded. UBZ: It was a narrow band of tissue extended about $\frac{3}{4}$ of the way around the circumference of the endometrium separating the decidual of SDZ from the inner circular layer of the myometrium.

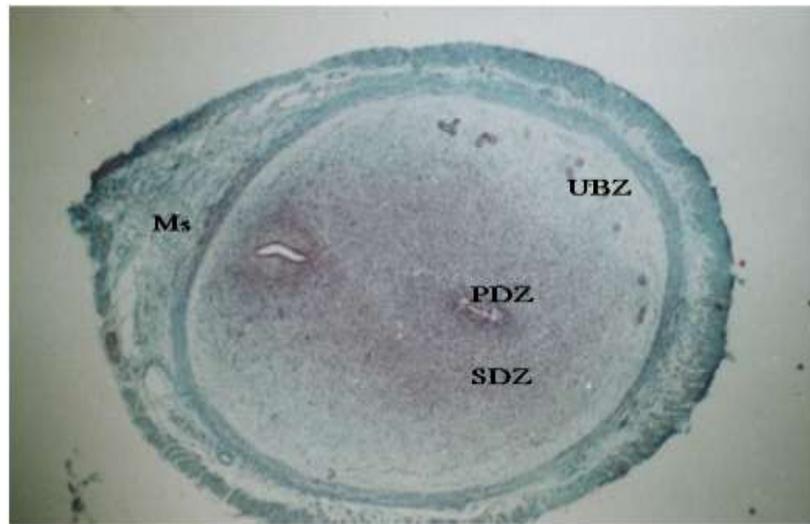
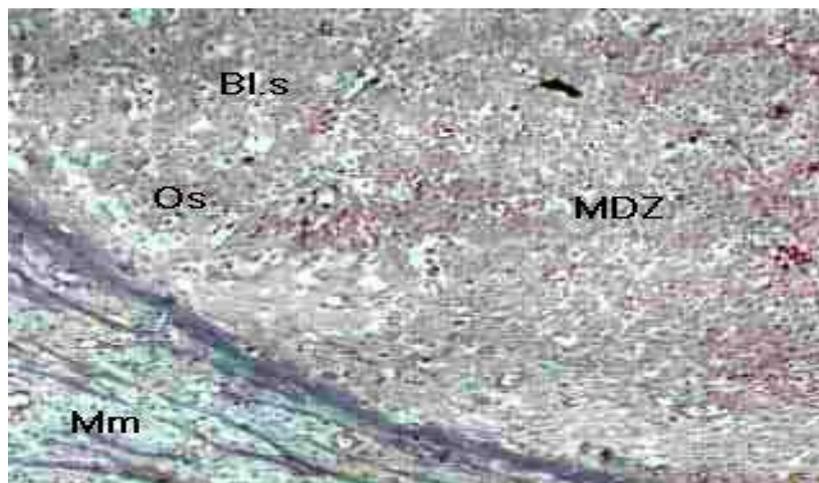


Figure 4: Cross section in Rat uterus on day 7 dpc; (Therap.Metro.27mg = female rats were treated with triple therapeutic dose). Showing the distribution of collagen fibers in the different zone of decidual tissue. The collagen fibers were absent from PDZ, present in minimal amount in SDZ, but the fibers were densely accumulated in the UBZ; Ms = mesometrial side of endometrium.. Gomori's one step trichrome stain. X20.



Figur 5 : Cross section in Rat uterus on day 9 dpc(subgroupTherap. Metro.9mg = female rats were treated with therapeutic dose of the Metronidazole). Collagen fibers is only present around the blood sinusoids (Bs) of the mesometrialdecidual zone (MDZ). It is absent from the outer places of decidual tissue collagen fiber is only seen at the outskirts (Os) of the DZ adjacent to the myometrium (Mm). Gomori's one step trichrome stain. X100.

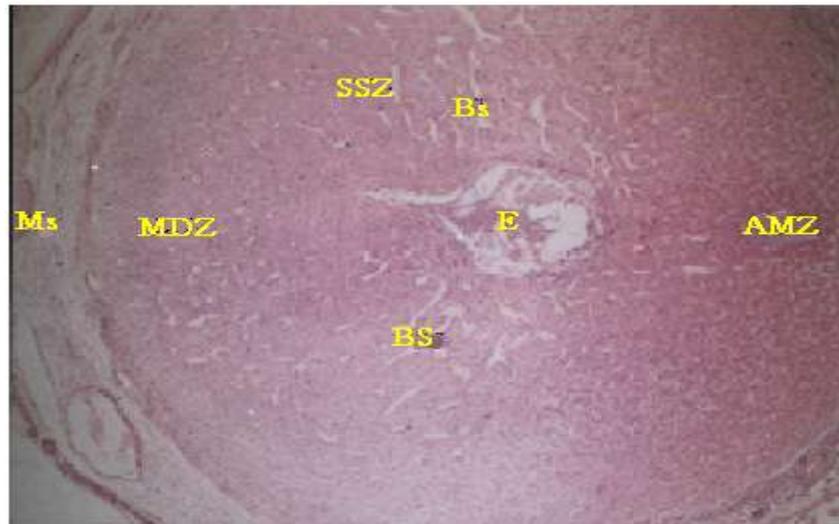


Figure 6 : Day 9 dpc (Therap. Metro. subgroup = female rats were treated with double therapeutic dose).A section of the uterus from treated rat, passing through the implantation site. Notice the rotation of the embryo (E) mesometrially. Blood sinusoids (BS) radiating toward the mesometrial side (Ms) where larger blood vessels are there.MDZ= mesometrialdecidual zone ; AMZ=Antimesometrialdecidualzone;SSZ=spiny shaped decidual zone. Hematoxylin and Eosin stain. X 40.

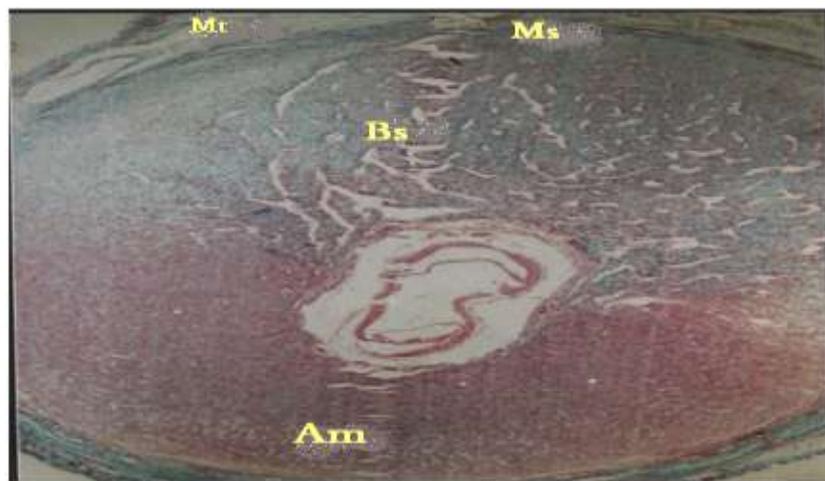


Figure 7: Cross section in the implantation site of rat stained for collagen fibers(green color). Day 9dpc (Therap.Metro. subgroup = female rats were treated with triple therapeutic dose of theMetronidazole) . Notice the uneven distribution of the fibers. They are either absent or sparse on the antimesometrial side (Am), but widely distributed in between decidual tissue and extensively branched and tortuous blood sinusoids (Bs) on the mesometrial side (Ms) of endometrium. The blood sinusoids are continuous with larger blood vessels in the mesometrial triangle (MT) ; E= embryo. Gomori's one step trichrome stain .X100.

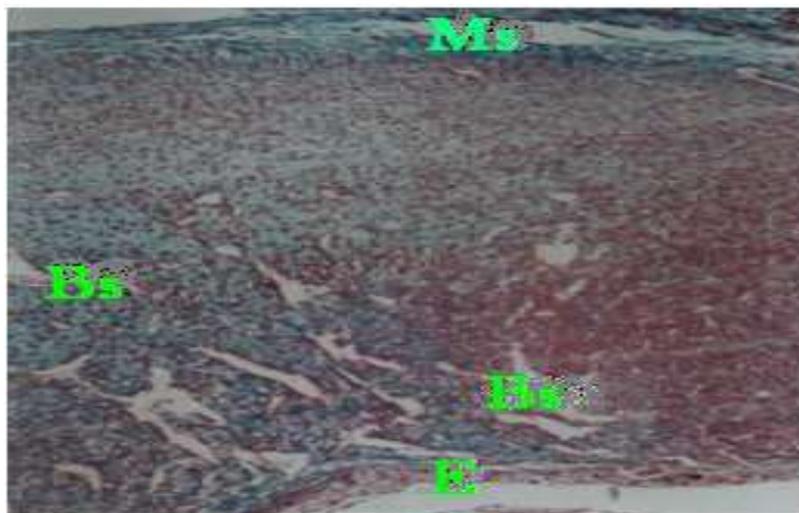


Figure 8: Higher magnification for a cross section taken from same area of figure 7. Notice the wide distribution of collagen fibers (green color) in between the blood sinusoids (BS) and decidual tissue of mesometrial side of the .100endometrium (Ms). Segment from the embryo (E) also shown . Gomori's One step trichrome stain . X

The Gomori's rapid one step trichrome stain had revealed the distribution of collagen fibers in the endometrium and the decidual tissue. The embryo was first located in opposition to the antimesometrial luminal epithelium. This area correlates with the region in which decidualization has been initiated, then progressed mesometrially.

In the PDZ the extracellular matrix was devoid of collagen fibers, mesometrially and antimesometrially (Fig.2). The area with minimal amount of collagen fibers corresponds to the differentiating tissue of SDZ. This zone extends around the mesometrial aspect of the uterine lumen and incorporates an area of marked edema antimesometrially (Fig.3). Dense accumulation of collagen fibers was seen in the stromal tissue of UBZ (Fig. 4).The collagen fibers of this zone were arranged in a form of whorl around the inactive endometrial glands.

Day 9 dpc (G2 group)

The implantation sites at day 9 dpc were exhibiting very clear bead-like appearance in which decidualization was in the highest degree of growth and development. In Hematoxylin and Eosin stained sections, the endometrium could be divided into five main zones (Fig. 6):

1. Decidual crypt zone (DCZ): Was located at the antimesometrial pole and in direct contact to the embryo. The decidual cells of this zone were closely packed to form the wall of the crypt.
2. Antimesometrial zone (AMZ): Constitute a zone of tightly packed decidual tissue in the antimesometrial side of endometrium. It was extending from the DCZ toward the nondecidulized tissue the undifferentiated basal zone (UBZ).
3. Undifferentiated basal zone (UBZ): A zone of nondecidulized, undifferentiated stromal cells, located between the myometrium and the fibrinoid capsule.

The cells of this zone resembled the fibroblast- like cells of the original endometrium. They have wide extracelullar space.

4. Mesometrialdecidual zone (MDZ): The MDZ was occupying a triangular area of Endometrium located between the myometrium and the mesometrial pole of embryo. The decidual cells of this zone were not densely packed as in ADZ. There was large tortuous blood spaces associated with this zone radiating from the mesometrial pole of the embryo toward the mesometrial triangle (Fig. 7).

5. Spiny-shaped decidual cells zone (SSZ): It was located lateral to the MDZ, and characterized by smaller sized cells with long numerous cytoplasmic processes. The extracelullar spaces in this zone were wide .

In Gomori's one step trichrome stain the collagen fibers were unevenly distributed within the different zones of decidual tissue and the endometrium. The collagen fibers were not displayed in the DCZ zone and the AMZ zone (Fig.6). But they were abundantly seen in the mesometrial triangle and the peripheral part of MDZ zone nearest to the myometrium, especially around the blood vessels of this zone (Figs. 6 and 7). Moderate amount of collagen fibers were seen in the SSZ zone. High condensation of collagen fibers within the UBZ zone were accumulated in between the undifferentiated stromal cells and the endometrial glands.

Discussion

One of the prominent results seen in the control and treated animals of this work was the presence of decidual reaction. Welsh and Enders ^[25] have pointed out that further expansion and successful development of the conceptus was closely tied to changes in decidua. By comparing the results of days 7 and 9 dpc, it was apparent that the endometrium has undergone marked changes reflecting a normal sequence of events seen in normal pregnancy of the rat.

Among many features, of PDZ demonstrated in the present work, was the absence of capillaries, tightly packing of their decidual cells, and absence of collagen fibers. In addition to that several kinds of intercellular junctions between cells of this zone have been described viz : tight junctions; gap junctions. These findings were suggested a barrier function of PDZ to the trophoblastic invasion during early stages of implantation.

Moreover, the cells of this zone could play a major role in maintaining coherence and structural support to the endometrium during early pregnancy as collagen fibers was absent in this zone.

Present observations regarding the presence of minimal amount of collagen fibers in SDZ was in agreement of Parr *et al.* ^[26]. The differing distribution of collagen fibers within PDZ and SDZ may reflect either the degree of decidualization within each area or distinct functional role. The cells of UBZ were not included in the process of decidualization. Thus, by day 7 dpc The UBZ is a wide band of loosely packed tissue. Subsequent growth of the decidual tissue zone at day 9 dpc leads to compaction of the UBZ with regularly arranged bundles of collagen becoming its major constituent.

The present study has demonstrated that one of the demarcation criteria between the decidualized tissue and nondecidualized tissue was marked by the distribution of collagen fibers. The PDZ was devoid of collagen fibers, while SDZ contained only minimal amount of collagen fibers. On the other hand, the UBZ was rich in collagen fibers.

The removal of collagen fibers from around the embryo at implantation site on day 7 dpc was closely related to the progressive steps of decidualization which was necessary for successful implantation and pregnancy. In the present work, no difference was noted between control and treated rats with regards to the distribution of collagen fibers. Tung *et al.* ^[27], and Clark *et al.* ^[28] have reported the absence of collagen fibers from decidual tissue and suggested that cellular adhesion between decidual cells allow to form an immunological barrier protecting the embryo from the mother immune response. If such concept was correct the absence of collagen fibers in decidual tissue during implantation may allow for necessary remodeling needed for establishment of decidual tissue. However the presence of minimal amount of collagen fibers in SDZ may be a stimulus for vasculogenesis. It had been shown that collagen fibers were a stimulating factor for vascular tube formation in vitro. The blood vessels have been noticed to be present in SDZ of the present work and previous studies ^[28;29;30;31;32]

Collagen fibers are likely to be important for support of placental vessels which will be functioning at day 8 dpc in the rat ^[33]. In this regards sections of day 9 dpc of this study displayed area with abundant amount of collagen fibers in the peripheral parts and around the blood vessels of MDZ nearest to the myometrium and in the mesometrial triangle. These two areas represent the prime route for establishment of nutrient supply via maternal blood vessels to supply the chorioallantoic placenta of the rat. This correlates with many reports ^[34;35;36;37] that the mesometrial

sinusoids radiating out from the mesometrial aspect of implantations chambers act as a venous system. Fainstat^[38] had explored the disappearance of collagen fibers in the early stage of pregnancy in rat. He concluded that, these fibers broke up into finer filaments during implantation and suggested that local decidual collagenase action released by growing decidua was responsible^[39;40;41;42]. Recently, it has been shown that the process of degradation of collagenous as well as noncollagenous components of extracellular matrix is involving a class of enzymes called matrix metalloproteinases (MMPs)^[39;42]. The MMPs enzyme has been localized in the cytotrophoblast cells which migrated to the endometrium during early pregnancy in human^[43;44;45], and Macaque^[46]. The disappearance of collagen fibers from some parts of decidual tissue of the rats in both the control and treated groups raised the question of the involvement of MMPs enzymes released by migrating cytotrophoblast cells in this process. Although the migration of cytotrophoblast cells has been exhibited in the rat^[39], MMPs enzymes localization in the migrating cytotrophoblast in this animal has yet to be elucidated.

Our results have revealed that, the oral administered Metronidazole (Flagyl[®]) drug, have not interfered with implantation, decidualization, and rearrangement of collagen fibers seen on days 7 and 9 dpc, which are essential for successful early pregnancy not just in the rat but in all types of hemochorial placentae. However, the use of Metronidazole in late pregnancy can cause teratogenic effect^[1;9].

In view of the results of the present work and previous studies, we can advise women in fertile period of life to use Metronidazole (Flagyl[®]) with caution especially during ovulation and implantation period of fetus^[47]. Further studies are required to elucidate the actual effects of long term use of Metronidazole (Flagyl[®]).

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