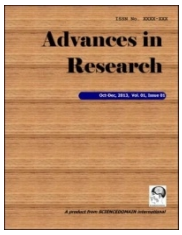

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The Status of Interstitial Cells of Cajal in Fallopian Tubes with Ectopic Pregnancy

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Authors' contributions

This work was carried out in collaboration between all authors. Author MEB designed the study, wrote the protocol, supervised the work and revised the manuscript. Author IK carried out the laboratory work, analyzed the data and wrote the first draft of the manuscript. Author SVN contributed to the laboratory work and revised the manuscript. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aims: To study the numbers and distribution of interstitial cells of Cajal (ICCs) in fallopian tubes (FTs) with ectopic gestation in comparison to normal FTs.
Study Design: Interstitial cells of Cajal were studied in normal FTs and FTs with ectopic gestation by immunohistochemistry using anti c-kit antibody.
Place and Duration of Study: Imperial College London, Department of Histopathology, Hammersmith Hospital, London, between April 2012 and August 2012.
Methodology: In this study we investigated the numbers and distribution of interstitial cells of Cajal in 50 FTs with ectopic gestation and in normal FTs from 25 patients. Interstitial cells of Cajal were highlighted by immunohistochemistry using anti c-kit antibody.
Results: Numbers of ICCs were significantly reduced in the muscularis and lamina propria of FTs with ectopic gestation as compared to normal FTs (P value: <0.001). In FTs with ectopic gestation the numbers of ICCs were significantly reduced at the implantation site when compared to areas away from the implantation site (P value = 0.003). There was no significant change in ICC numbers or distribution with patient age.
Conclusion: There is significant decrease in numbers of ICCs in FTs with ectopic gestation, which may compromise the motility of the FTs and hence transport of the

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gametes and embryo to the uterine cavity, predisposing to tubal implantation and ectopic gestation.

Keywords: Interstitial cells of Cajal; fallopian tube; ectopic pregnancy.

1. INTRODUCTION

Ectopic pregnancies occur in approximately 1.5-2% of pregnancies [1] and result in 10% of all pregnancy related deaths [2]. In ectopic pregnancy, the embryo can be implanted within the adnexa of the uterus, such as the fallopian tubes (FTs), ovaries, or into the cervix, the interstitial tubal segment, and other intra-abdominal sites [3]. Ectopic tubal pregnancies are the most common type, representing more than 98% of ectopic pregnancies [4].

The FT is involved in gamete and embryo transport to the uterine cavity, and is in addition the site of sperm capacitation, fertilisation and pre-implantation development of the embryo [5]. Oocyte transport through the FT is mediated by peristaltic action of the circular and longitudinal muscles of the FT walls which begin to contract rhythmically prior to ovulation. In addition, it is aided by the synchronous beating of cilia and a current of fluid produced through the action of the ciliated epithelium [6]. Sperms migrate up against this current from the cervix and to the site of fertilisation, and in a normal intrauterine pregnancy the zygote is transported from the ampullary region and to the uterus where it will implant and mature [7]. In a tubal ectopic pregnancy, the zygote will not be transported to the uterine cavity, and will implant and mature within the FT [4,6].

Interstitial cells of Cajal (ICC) were named after Santiago Ramon y Cajal, who first reported them in the gastrointestinal tract at the end of the 19^{th} century [8]. In addition to the gastrointestinal tract, ICC have been found in the mammary gland, myocardium, urinary tract, testes and FT [9]. ICC, which are electrically coupled to each other and to neighbouring smooth muscle cells via gap junctions [10], have been proven to act as pacemaker cells in the gut musculature [11]. ICC generate electrical activity in the form of slow waves, which is then conducted to the smooth muscle syncytium, producing spontaneous contractions of the muscle layer [10]. Smooth muscle cells do not generate or propagate slow waves themselves, but express voltage-dependent ion channels such as Ca^{2+} channels which respond to slow wave depolarisations [10]. It has been shown that Ca^{2+} release from inositol trisphosphate (IP_3) receptor-operated stores in ICC is linked to the initiation of pacemaker currents [12]. The release of Ca^{2+} from IP_3 receptors triggers mitochondrial uptake of Ca^{2+}, which lowers Ca^{2+} activity in the space between the plasma membrane and mitochondria. This reduction in Ca^{2+} activates Ca^{2+} inhibited non-selective cation conductance, resulting in an inward pacemaker current causing depolarisation. The waves of depolarisation spread from primary ICC to secondary ICC, activating voltage-dependent Ca^{2+} channels in the secondary ICC as well as in adjacent smooth muscle cells, generating rhythmical contractions of the smooth muscle [12,13].

Studies of isolated smooth muscle cells have not recorded slow wave activity [14], whereas studies of cultured ICC have shown spontaneous electrical activity similar to slow waves recorded in intact muscle [15]. In addition, studies have shown that slow waves in ICC precede the activity of neighbouring smooth muscle cells [16], supporting the hypothesis that ICC are the source of slow waves.

The presence of ICC has been demonstrated in the human FT. They are localised mainly in the connective tissue of the mucosal lamina propria, muscularis and subserosa [17,18]. It has been shown that FT ICC in culture exhibit spontaneous electrical activity in the form of short potentials without a rhythmical pattern. This, along with the presence of numerous gap junctions and Ca^{2+} release units suggest a pacemaker role for ICC in the human FT [17].

It has also been shown that ICC are involved in enteric inhibitory neurotransmission between nerve terminals and smooth muscle cells (SMCs) as they have been found to be intercalated between nonadrenergic, noncholinergic nerves and SMCs in the muscle layers of the gastrointestinal tract [19]. Neurotransmitters released from enteric motor neurones bind to receptors on ICC and ICC are responsive to several enteric transmitters including nitric oxide and acetylcholine. Loss of ICC networks in the stomach of mice has been found to result in loss of nitric oxide neurotransmission [20].

As the absence, reduction in numbers or altered integrity of ICC in the GI system can have an effect on gastrointestinal tract motility, Shafik and colleagues suggested that the absence or deficiency of ICC in the human FT may also have an effect on motility and therefore interfere with gamete and embryo transfer through the FT [21]. This can cause retention of the embryo within the FT, resulting in an ectopic pregnancy.

In this study we investigated the distribution and numbers of ICC in the muscularis and lamina propria of normal FTs in comparison to FTs with ectopic gestation. We also compared the number of ICC in FTs with ectopic gestation at the implantation site and away from it.

2. MATERIALS AND METHODS

2.1 Case Selection

Fifty cases of FTs with ectopic gestation and twenty-five normal FTs were obtained from the Department of Histopathology, Hammersmith Hospital, London, UK. Sections from all specimens were examined to select a representative block for the study and to exclude the presence of another pathology, as inflammation, infection or neoplasia. The normal FTs were obtained from specimens of total abdominal hysterectomy and bilateral salpingo-oophorectomy for uterine or ovarian lesions. All sections from the FTs were reviewed to confirm normal histological features and absence of any pathology.

Approval for the use of human tissue was acquired from the Institutional review board of the Human Biomedical Research centre at the Imperial College Healthcare NHS trust.

2.2 Immunohistochemistry

ICC in FTs were highlighted by immunohistochemistry (IHC) using anti c-kit antibody. IHC was performed on formalin fixed paraffin embedded tissue sections.

The sections were dewaxed in xylene, rehydrated in descending grades of ethanol then water and then incubated in 0.6% hydrogen peroxide for 15 minutes to block endogenous peroxidase activity. Antigen retrieval was performed by microwaving the sections in citrate (0.01M, pH6) buffer for 20 minutes at 700 W. The slides were rinsed in cold water, then incubated with protein block for 5 minutes. This was followed by a 5 minute wash in 0.01%

PBS/Tween and then the slides were incubated with the primary antibody (at a 1/400 dilution) overnight at 4ºC. The sections were washed then incubated with post-primary blocking buffer for 30 minutes. The buffer was rinsed off with 0.01% PBS/Tween and replaced with peroxidase polymer linked secondary antibody for 30 minutes. The sections were subsequently washed, developed with hydrogen peroxide and 3, 3-diaminobenzidine (DAB), rinsed and counter-stained with haematoxylin. The sections were then dehydrated in ascending grades of alcohol, cleared in xylene and mounted.

A positive control slide (colon section) was stained with every run of IHC staining. An additional slide of FT specimen for each run was also included as a negative control, which was treated with antibody diluent instead of the primary antibody.

The primary antibody was obtained from DAKO UK Ltd. (Ely) and the secondary antibody and other reagents were provided in a Novolink polymer kit (Leica UK Microsystems, Milton Keynes).

2.3 Assessment of Staining

The distribution and number of ICC were assessed in the muscularis layer of 5 non-overlapping low power fields and in the lamina propria of 3 non-overlapping low power fields, using a light microscope (Olympus CH series) at a magnification of x 100 (using X10 objective lens). ICC expression was also assessed in the muscularis of FTs with ectopic gestations at the implantation site, and away from the implantation site, in 5 non-overlapping low power fields. Mast cells are also highlighted by c-kit antibody, but are morphologically different from ICC. Only cells expressing c-kit and showing the morphology of ICC were counted.

2.4 Statistical Analysis

Statistical analysis was performed using Mann-Whitney U-test. Mean values ± standard deviation were expressed and a probability (p) value of <.05 indicated statistical significance. Statistical analysis was performed using SPSS (version 16.0, Chicago, IL, USA).

3. RESULTS

3.1 ICC in Normal FTs

ICC were found within the lamina propria of the mucosa (Fig. 1A) and within the longitudinal and circular muscle layers (Fig. 1B) of normal FTs. In the muscularis, ICC were surrounded by and in parallel orientation with SMCs, and did not appear to form a clear network. ICC tended to be distributed towards the inner border of the circular muscle layer, near the interface between the circular muscle layer and submucosa, and in between the circular and longitudinal muscle layers. The mean number of ICC in the muscularis of normal FTs was 16.64 (±4.94) per low power field (Table 1). ICC appeared to be more frequent in the circular than in the longitudinal muscle layer. The mean value of ICC numbers in the normal FTs was 11.84 (±4.68) per low power field (Table 2).

Table 1. Mean ICC counts (± standard deviation) per low power field in the muscularis layer of normal FTs and FTs with ectopic gestation

Group	N	Mean ± SD	P-value
Normal	25	16.64±4.94	<.001
Ectopic	50	6.53±3.08	

3.2 ICC in FTs with Ectopic Gestation

In FTs with ectopic gestation ICC were found in the same locations as in normal FTs, although the numbers were notably reduced when compared to normal FTs (Fig. 1C and 1D). The mean number of ICC in the muscularis of FTs with ectopic gestation was 6.53 (± 3.08) per low power field (Table 1). There was a 60.8% reduction in ICC numbers in the muscularis of FTs with ectopic gestations in comparison to the muscularis of normal FTs, which is statistically significant (p value <.001).

In the lamina propria, mean value of ICC numbers was 7.39 (±3.79) per low power field, which significantly less than ICC numbers in normal FTs (p value <.001) (Table 2).

Table 2. Mean ICC counts (± standard deviation) per low power field in the lamina propria of normal FTs and FTs with ectopic gestation

Group	N	Mean ± SD	P-value
Normal	25	11.84±4.68	<.001
Ectopic	35	7.39±3.79	

A reduction in the number of ICC was found in the muscular layer of FTs with ectopic gestation at the implantation site, in comparison to the muscular layer away from the implantation site (Fig. 1D and E). The mean number of ICC in the muscularis at the implantation site was 7.86 (±5.08) per low power field and 12.85 (±4.54) per low power field away from the implantation site, showing a statistically significant reduction at the implantation site (p-value .003) (Table 3).

Table 3. Mean ICC counts (± standard deviation) per low power field in the muscularis layer of FTs with ectopic gestations, at and away from the implantation site

Group	N	Mean ± SD	P-value
At implantation site	13	7.86±5.08	.003
Away from implantation site	13	12.85±4.54	

3.3 ICC Number in Relation to Patient Age

Changes in ICC numbers in the muscularis and lamina propria of normal FTs and FTs with ectopic gestation were assessed against patient age. No significant change in ICC numbers was identified in relation to patient age in normal FTs or FTs with ectopic gestation in either the muscularis layer or the lamina propria (Table 4).

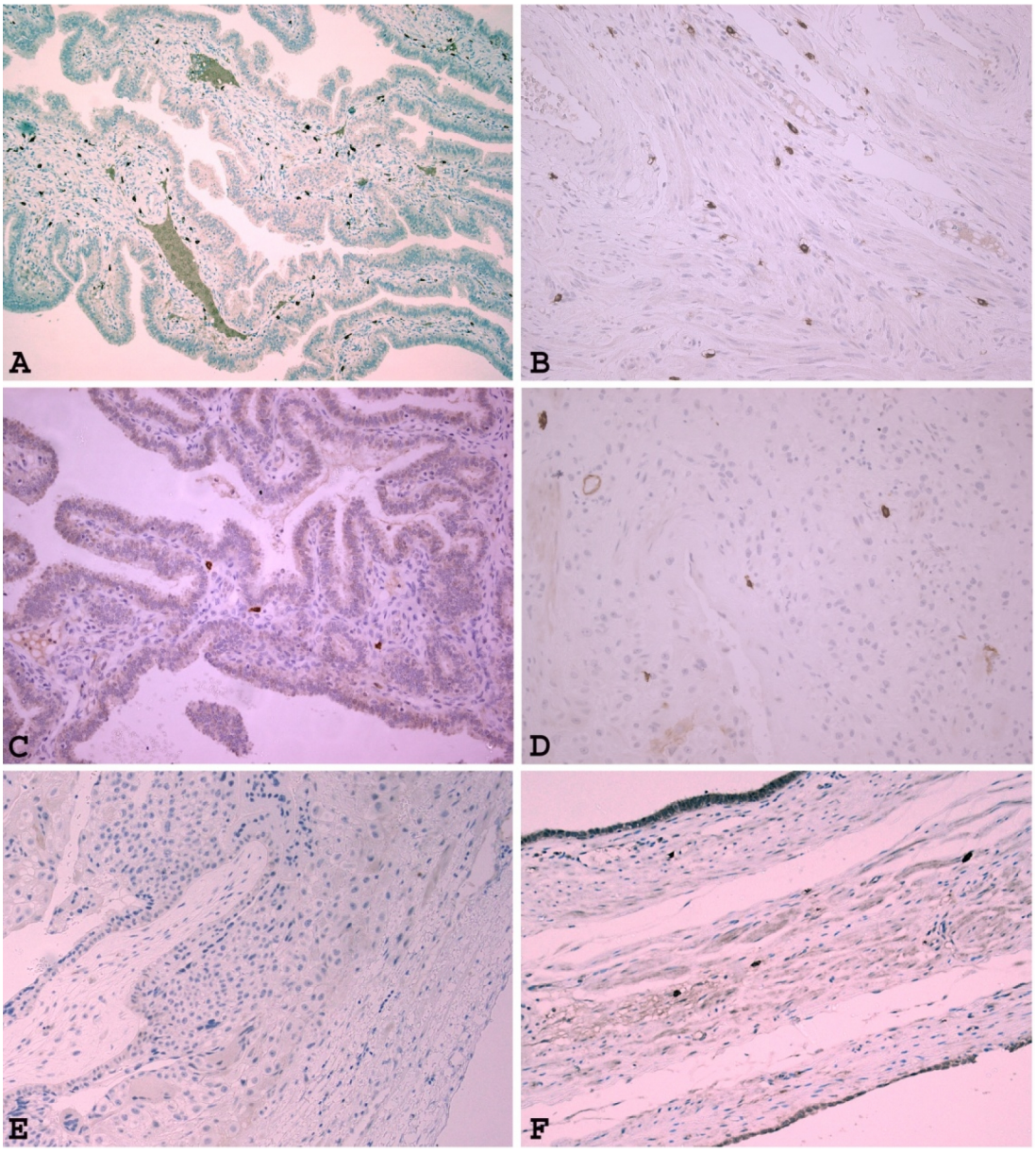

Fig. 1. ICC in normal FTs and FTs with ectopic gestation. ICC appear as round to elongated brown bodies. (A) ICC in the lamina propria of normal FTs. (B) ICC in the muscularis layer of normal FTs. (C) ICC in the lamina propria of FTs with ectopic gestation; significantly less numbers as compared to normal FTs. (D) ICC in the muscularis layer of FT with ectopic gestation; significantly less numbers as compared to normal FTs. (E) Implantation site in FT with ectopic gestation; no ICC are seen. (F) FT with ectopic gestation away from implantation site; few ICC are seen. (Magnification X200)

Table 4. Mean ICC counts per low power field (± standard deviation) in the muscularis layer and lamina propria of normal FTs and FTs with ectopic gestation in relation to patient age

Age	Normal		Ectopic	
	Muscularis	Lamina propria	Muscularis	Lamina propria
20-29	19.3±3.5	11.34±0.335	6.54±3.77	7.63±3.88
30-39	17±2.6	20.83±8.5	6.64±2.65	6.99±3.93
40-49	14±1.77	13±2.38	6.65 2.86	9.56±2.67
50-59	16.07±6.95	12.44±3.39	-	-
60+	16.45±4.6	9.58±2.18	-	-

4. DISCUSSION

Our study shows significant decrease in the numbers of ICC in FTs with ectopic gestation. The FT plays a central role in the process of human reproduction. Prior to ovulation, the FT begins to contract rhythmically due to peristaltic action of the circular and longitudinal muscles and along with ciliary movements; these actions transport the oocyte to the site of fertilisation and the embryo to the uterine cavity where it will develop [22]. However, in a tubal ectopic pregnancy, the embryo is implanted within the FT [23].

It has been suggested that ICC in the human FT act as pacemaker cells, conducting electrical activity to smooth muscle cells [21], as has been long known in the gastrointestinal tract [11] and are therefore involved in FT motility [21]. The peristaltic action of the longitudinal and circular muscle in the human FT is thought to be initiated by ICC and conducted to the smooth muscle cells via gap junctions [17].

The study by Ahmed et al. [18] is the only published study which investigated the distribution of ICC in normal FTs in comparison to FTs with ectopic gestation. Ahmed and colleagues assessed ICC within the muscularis layer and lamina propria of 20 normal FTs and 20 FTs with ectopic gestation, and found a significant reduction in ICC numbers in the different layers of the walls of FTs with ectopic gestation. In this study we examined a larger number of FTs with ectopic gestation (50 cases) and 25 normal FTs and in addition also studied the numbers of ICCs near and away from implantation site in FTs with ectopic gestation. Our results in agreement with the findings of Ahmed et al. show significant reduction of ICC numbers in the muscularis layer and lamina propria of FTs with ectopic gestation in comparison to normal FTs. In both studies, ICC appeared to be present in higher numbers in the circular muscle layer than the longitudinal muscle layer. This could explain the higher frequency of contractions of the FT in the circular muscle layer than the longitudinal muscle layers, as described by Helm et al. [24]. ICC did not appear to form a clear network, which has also been found to be the case in the vas deferens [25].

It has been shown that ICC in the human FT are closely related to nerve fibres where they integrate the signals between nerves and SMC [26]. ICC express receptors for neurotransmitters which elicit electrical activity in ICC which is then conducted to smooth muscle cells. ICC therefore act as intermediates in enteric inhibitory neurotransmission [27]. It has also been confirmed that ICC in the human FT exhibit spontaneous electrical slow wave activity which is conducted to neighbouring smooth muscle cells via gap junctions [17]. In the gastrointestinal tract, smooth muscle cells respond to this slow wave activity by activation of voltage-dependent Ca^{2+} channels. This results in spike-like Ca^{2+} action

potentials in some regions and in other regions an increase in the open probability of the Ca^{2+} channels. In both regions these then result in an influx of Ca^{2+} and excitation-contraction coupling of the smooth muscle layers [28]. It is possible that the reduction in ICC numbers in FTs with ectopic gestation as seen in the present study, are either resulting in loss of neurotransmission between nerve terminals and smooth muscle cells through ICC or in a reduction of electrical activity along the FT. These events could then be slowing down or preventing gamete or embryo transport to the uterine cavity, causing embryo retention and implantation within the FT.

ICC in the FT have been found to express progesterone and oestrogen receptors, whereas ICC in contrast with ICCs in other organs [29]. There is therefore also a possibility that ICC in the FT function as steroid hormone sensors by responding to progesterone and oestrogen levels through gap junctions or juxtacrine and/or paracrine mechanisms, which then influence smooth muscle cell activity and therefore FT peristalsis [30]. Perhaps the loss of ICC in FTs with ectopic gestation results in less hormone dependent activity, affecting the peristalsis of the FTs and contributing to the detainment of the embryo within the FT.

We found a statistically significant reduction in ICC numbers in the lamina propria of FTs with ectopic gestation compared to normal FTs (p-value <.001). This reduction is in line with the findings of Ahmed *et al*. who also found that the numbers of ICC in the lamina propria were significantly reduced in FTs with ectopic gestation [18]. Smooth muscle cells are not present within the lamina propria, and therefore a pacemaker role for ICC within this location is unlikely. However, the lamina propria is under the control of steroid hormones [31] and as already mentioned, ICC express progesterone and oestrogen receptors. It has been suggested that ICC in the lamina propria influence mucosal ciliary movement depending on progesterone and oestrogen levels [29]. It has also been suggested that ICC within the lamina propria of the rat jejunum may act as stem cell adjutants, involved in renewal of the epithelium [32]. The epithelium within the lamina propria of the FT is ciliated, and the cilia are thought to have some role in the motility of the FT. It is possible that the reduction of ICC in the lamina propria of FTs with ectopic gestation is resulting in a reduced rate of renewal of the ciliated epithelium, therefore affecting the transport of the gametes and embryo through the FT and to the uterine cavity.

Our study is the first to investigate the distribution and number of ICC in FTs with ectopic gestation at the site of implantation and away from the implantation site and we found that there was a significant reduction in ICC at the implantation site compared to away from it. This is the first study to investigate the numbers of ICC at different sites in FTs with ectopic gestation. Our results show a significant reduction in ICC numbers at the site of implantation compared to away from it (p-value: .003), with a mean difference of 5.94 (±4.42) cells per low power field.This raises the question of whether implantation of the embryo within the FT affects the local distribution and population of ICC. However, although there was a reduction in ICC numbers at the implantation site compared to away from the implantation site, there was a significant, overall reduction in ICC numbers in segments of FTs with ectopic gestation at the implantation site as well as away from it compared to normal FTs. This suggests that it is not simply the presence and implantation of the embryo within the segment of the FT which is causing the reduction in ICC numbers, although it is likely that it does have some effect on ICC in that area. It is also possible that the excessive loss of ICC at this point reduces peristalsis at and beyond this segment of the FT even further, enhancing the chances of implantation of the fertilised ovum at this site. In addition to confirming the findings reported by Ahmed et al., the present study also reports a significant reduction in ICC in FTs with ectopic gestation at the implantation site compared to away

from the implantation site. To the best of our knowledge, this finding has not been reported before.

The incidence of ectopic pregnancy has been shown to increase with increasing maternal age [33]. In 2011, Gomez-Pinilla and colleagues reported a decrease in ICC numbers in the human stomach and colon with increasing patient age [34] and based on their findings suggested that the number of ICC in the FTs could be declining with age, resulting in loss of FT motility, causing the increase in the incidence of ectopic gestation seen with increasing maternal age. In this study we investigated whether increasing age had an effect on ICC number and distribution in FTs, which has not been previously studied. No relationship was found between age and number of ICC whether in normal FTs or FTs with ectopic gestation.

This is the largest and most detailed study to date of the numbers and distribution of ICC in FTs with ectopic gestation and the first study to assess ICC at and away from implantation site and the relationship with patient age. In conclusion, the statistically significant reduction in ICC numbers found in both the muscularis and lamina propria of FTs with tubal ectopic gestation – both at the implantation site and away from the implantation site - in comparison to normal FTs suggests that the reduced number of ICC may be a cause of compromised tubal motility, hindering transport of the embryo to the uterine cavity, causing it to implant within the FT.

5. CONCLUSION

There is significant decrease in numbers of ICCs in FTs with ectopic gestation, which may compromise the motility of the FTs and hence transport of the gametes and embryo to the uterine cavity, predisposing to tubal implantation and ectopic gestation**.**

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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