

denatured biotin-labeled DNA probe diluted in 9.2 μ l hybridization solution) per slide. After overnight incubation, the slides were soaked for 10 minutes in 1X detergent wash at 37°C, followed by RNase A treatment at 37°C for 30 minutes, and then the slides were washed for 5 minutes in 1X protein blocking buffer. The biotin-labeled hybrids were detected with streptavidin-alkaline-phosphatase conjugate, and an enzyme-substrate chromogen (bromo-chloro-indolyl-phosphate/ in nitro-blue-tetrazolium salt) BCIP/NBT, yielding an intense blue-black signal appears at the specific site of the hybridized probe. The slides were counterstained with nuclear fast red stain. (Poor tissue quality or target RNA degradation may give false negative results or poor signal. This could be verified by using a probe to an abundant RNA target like the probe of a housekeeping gene which is a sequence or gene product that is constitutively expressed in most tissue types such as actin or tubulin. The specificity of the ISH signal was assessed by: 1) RNase A treatment of the tissue sections for 2 hours at 37 °C, before the *in situ* hybridization, and 2) omission of the probe in the hybridization mixture).

Evaluation of ISH signal: The expression of IFN- γ and IL-10 mRNAs was measured by counting the number of positive decidual and trophoblastic cells, which gave a blue-black (BCIP/NBT) nuclear staining under the light microscope. The extent of the ISH signal in the villi was determined in 10 fields (X100 magnification). In each field the total number of villi were counted and the extent of nuclear staining of the cytotrophoblast and syncytiotrophoblast in a given villous was graded as 3, (75–100%); 2, (25–75%); or 1, (<25%). The total staining score was divided by the number of

whole villi per field in 10 fields. These scores (between 1 and 3) were added for each field, and a score between 10 and 30 was gained for each sample. The scorer was blinded to the clinical diagnosis of the tissues at the time of assessment, and tissues were independently assessed by two observers, and as advised by Hennessy (Personal communication, 2004). For more details, refer to the *In situ* hybridization procedure and signal evaluation in references ⁽²⁶⁻²⁷⁾.

Statistics:

ANOVA test was used to determine the difference in the *in situ* expression of IFN- γ or IL-10 among the three groups and in between each two groups, and the relationship between these two parameters was measured using the correlation coefficient (*r*). Values of $p < 0.05$ were considered as statistically significant ⁽²⁶⁾.

Results

The expression of IFN- γ and IL-10 was detected by ISH technique, (Tables 1 and 2) show the percentages of IFN- γ and IL-10 *in situ* expression respectively in the villus trophoblasts in terms of mean \pm SE, median, minimum and maximum values of the three groups. (Table 3) shows the difference in the expression of IFN- γ and IL-10 among the three groups and within the groups using ANOVA analysis.

The study demonstrated no significant correlation between IFN- γ and IL-10 ($p = 0.23$, $r = 0.23$), however, the ratio of IFN- γ /IL-10 was 1.97 in women with recurrent abortion, while that of normal pregnant and first abortion groups were 0.67 and 0.73 in an order.

The expression of IFN- γ and IL-10 was heterogenous blue-black nuclear staining, involving both decidual and trophoblastic cells, as shown in (Figure 1).