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EFFECT OF TUMOUR NECROSIS FACTOR-α (TNF-α) ON PROTEIN SYNTHESIS BY MOUSE PREIMPLANTATION STAGE EMBRYOS *IN VITRO*

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Abstract : Successful blastocyst implantation depends upon the synchronous dialogue between age- and stage-matched embryo and adequately primed maternal endometrium. Endometrial signals present in the uterine lumen influence the growth and the viability of preimplantation stage embryo. Thus, uterine secretion of embryotoxic cytokines may affect the preimplantation stage embryo. Our previous study in the rhesus monkey has indicated that tumor necrosis factor-α (TNF-α) is one such candidate present in the uterine lumen, which may act as an embryotoxic agent. In the present study, the effect of TNF-α on *de novo* protein synthesis by mouse morulae (n=100) and blastocysts (n = 100) *in vitro* was investigated by 2D-polyacrylamide gel electrophoresis. A total of 35 and 40 protein spots were detected in lysates of control morulae and blastocysts, respectively. Exposure of embryos to TNF-α (50 ng/ml) reduced the number of protein spots to 15 and 17 compared to that of control morulae and blastocysts. Seven spots in morula and 13 protein spots in blastocyst flourograms showed quantitative changes in their expressions with exposure to TNF-α. Morulae and blastocysts exposed to TNF-α expressed 8 and 17 protein spots, respectively, that were not seen in control embryos. It appears from the present study that exposure of preimplantation stage embryos to TNF-α affects their protein synthesis both quantitatively and qualitatively.

Key words : 2D-electrophoresis blastocyst morula mouse protein TNF-α

INTRODUCTION

Preimplantation stage embryo development and successful implantation depends on protein factors synthesized and secreted by the endometrium into the uterine lumen under adequate hormone regulation (1–3). These molecules are largely regulated by progesterone (2–6). We have demonstrated in the rhesus monkey that inhibition of progesterone action by early luteal phase mifepristone administration

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results in inhibition in the morula to blastocyst transition, adversely affecting the viability of blastocysts and the process of implantation (7, 8). Thus, the adverse effect observed in the preimplantation stage embryo recovered from the uterine lumen of mifepristone treated animals may be attributed to the altered milieu of the uterine lumen. Among various biomolecules, tumour necrosis factor-α (TNF-α) appears to be one of the candidates as it has been reported that TNF-α is regulated in a cyclical manner in the human endometrium (9), and that progesterone inhibits endometrial TNF-α synthesis and secretion (2, 10). Thus, TNF-α may be responsible for the degenerative changes observed in the embryos recovered from the anti-progesterone treated animals (8). Indeed, several studies show that TNF-α decreases the viability, growth and differentiation of preimplantation stage blastocysts (11–15). In the present study, we have examined the effect of TNF-α on the functional characteristics of mouse morulae and blastocysts *in vitro* in terms of their *de novo* protein synthesis, as the pattern of protein synthesis could be a better functional marker for cell function (16).

The advancement of newer techniques have helped us in understanding the protein expression profile in an improved manner. Two dimensional electrophoresis (2D-electrophoresis) is one of such techniques, that has been used to a greater extent in terms of its reproducibility to study protein expression profile by protein mapping (17). Using this robust technique, proteins in a complex solution are resolved into individual protein spots based on their iso-electric points (*p*I) and molecular weight ranges (M*r*). In the present study, we employed 2D-electrophoresis to examine the protein profiles of morulae and blastocysts following their exposure to TNF-α.

METHODS

Mouse embryo collection and culture

Random-bred sexually mature Swiss albino female mice were subjected to superovulation and allowed to mate with adult males according to the standardized protocol (18). Day 1 of pregnancy was designated from the day of detection of copulatory plug. Uterine horns were flushed with M2 medium (pH 7.4) on day 4 morning (19), and morulae and blastocysts with normal appearance under phase contrast microscope were collected and used in different experiments as described below. Medium M16 (pH 7.6) supplemented with sodium pyruvate (36 μg% w/v), bovine serum albumin (0.4% w/v) along with or without TNF-α (50 ng/ml, R&D Systems, Minneapolis, USA) was used at a ratio of 50 μl/embryo to culture morulae and blastocysts taken in randomly assigned pools of 4 embryos. The study design was approved by the Institutional Animal Ethics Committee of the All India Institute of Medical Sciences, New Delhi.

Protein synthesis by mouse embryos

In order to examine the protein synthetic ability of morulae and blastocysts with or without TNF-α (50 ng/ml) *in vitro*, embryos (n = 100) were cultured for 4 h in M16 medium containing 100 μCi/ml of ^{35}S-methionine in humidified 5% carbon dioxide in air at 37ºC (20).

Embryos were washed and lysed in 25 µl of sample buffer containing SDS 0.30% w/v, DTT 3.09% w/v, Tris-HCl 0.44% w/v, Tris base 0.27% w/v. High amount of dithiothreitol and very less amount of SDS is sufficient to solubilise the cell membrane of embryonic cells without reducing the protein or cleaving the disulphide bonds. The mixture is vortexed briefly to complete the lysis. The lysed samples were stored at –70C till further use. The batch variation was taken care by subjecting all the samples together for iso-electric focusing followed by SDS-PAGE. Prior to loading the sample on to the iso-electric focusing gels, the samples were thawed completely to dissolve high amount of DTT present in the sample buffer and were subjected to centrifugation for 10 min at 12000 Xg. A small portion of the lysate (2 µl) was taken to measure the total radioactive count using scintillation counter and samples with a total count of 1 $X10^5$ CPM was loaded in the first dimensional gel, in glass tubes (ID: 1 mm; length:7 cm) containing 4% (w/v) acrylamide, 2.1% (w/v) Triton-X-100, 59.8% (w/w) urea and a mixture of ampholytes for creating continuous pH gradient of 3 to 7 the samples were subjected to iso-electric focusing at 500 V, 12 h using 100 mM phosphoric acid and 0.1 M NaOH as anode and cathode solutions in a Mini Protean II set up from BioRad (Bio-Rad, California, USA) according to the method described by Dunbar (21). After completing the iso-electric focusing the tube gels were extruded from the tubes and were reduced in sample buffer containing 3% (w/v) SDS , 0.8% (w/v) DTT, 1.18% (w/v) Tris-HCl, 3.63 % (w/v) Trisma base and 0.01% (w/v) bromophenol blue for 2 min. The second dimensional separation was carried out by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE; 12.5% acrylamide). 2D-molecular weight markers (Bio-Rad, California, USA) were run on a separate gel. After completing the slab gel, the gels (7 cm x 9 cm x 1 mm) were fixed in 50% methanol, 10% acetic acid for 30 min, treated with Amplify (Amersham Pharmacia Biotech, Amersham, UK) for 30 min, dried and exposed to Hyperfilm-β max (Amersham Pharmacia Biotech, Amersham, UK) for 30 days.

Analysis of 2D-gel flourogram images

Fluorograms of 2D gel electrophoresis were scanned in high resolution scanner (60 nm, Personal FX, Bio-Rad Laboratories) and converted to 8 bit 1024x1024 pixel image and stored as TIFF images. The images were analyzed using the analysis software, PDQest (BioRad, California, USA). Spot detection was carried out using algorithmic analytic tool, and spot integrated optical density of each spot on the individual gel was calculated. The original scans were filtered and gels were matched by using the software program with the help of key spots and master fluorogram and a three-dimensional Gaussian image was created from clarified Gaussian spots. A Gaussian spot is a precise three-dimensional representation of an original scanned spot that is filtered and clarified from noise and background. The spots, which were not included in the Gaussian image after filtration, were not included for further analysis. A matchset of the gels was prepared from the Gaussian image and a cartoon image was created for visual display. For quantitative analysis of 2D gels, spots in the fluorograms with relatively weak and strong intensity were selected so that intensities of the spots could be fixed in a linear curve for quantification. The

scanned fluorgrams had comparable total scanning density with a coefficient of variation of 14%. For comparison between the fluorograms of a given spot, the data were noted as spot integrated optical density relative to the total density of the individual fluorogram (IOD%).

RESULTS

Figures. 1 and 2 show the representation of fluorograms of ^{35}S-methionine labeled

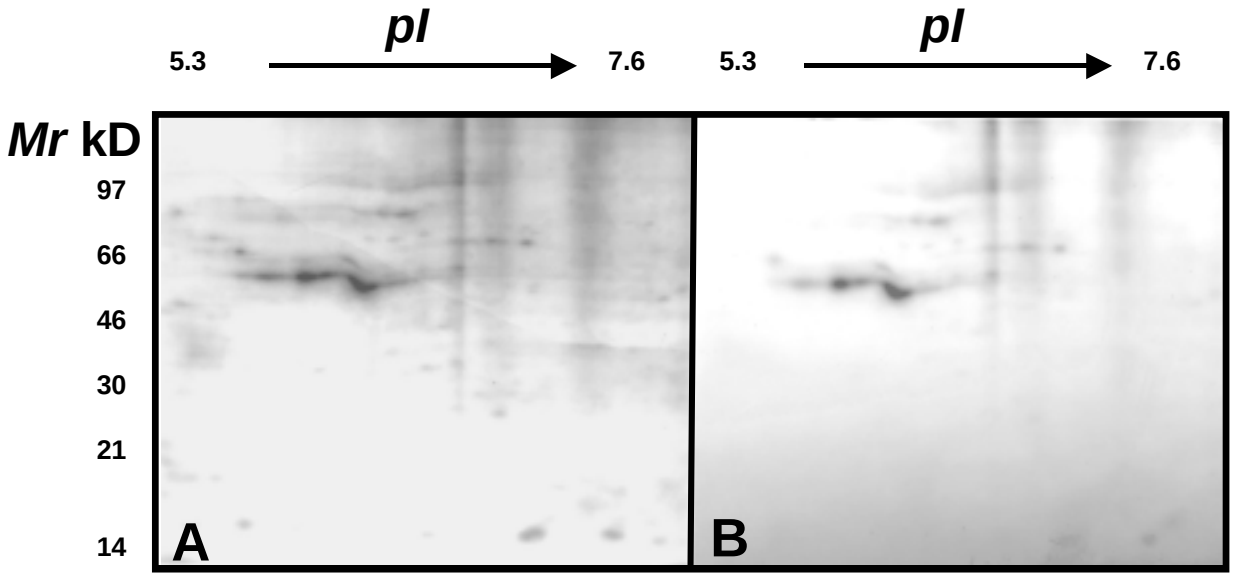

Fig. 1 : Images of flourograms of *de novo* synthesized proteins of mouse morula without (A) and with exposure of TNF-α, 50 ng/ml (B).

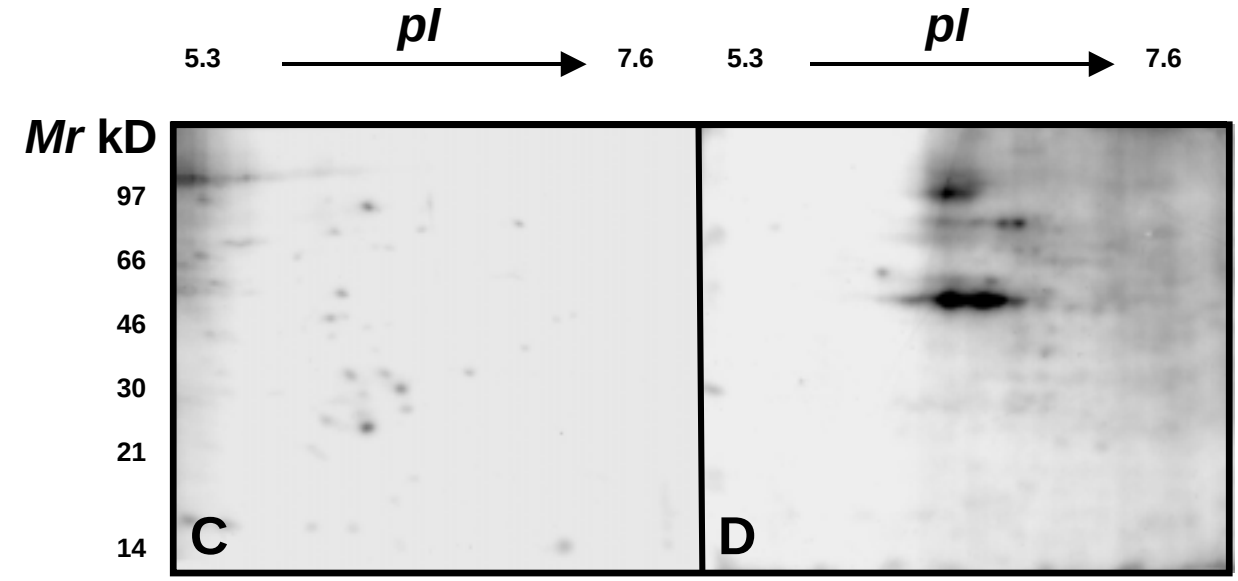

Fig. 2 : Images of flourograms of *de novo* synthesized proteins of mouse blastocyst without (A) and with exposure of TNF-α, 50 ng/ml (B).

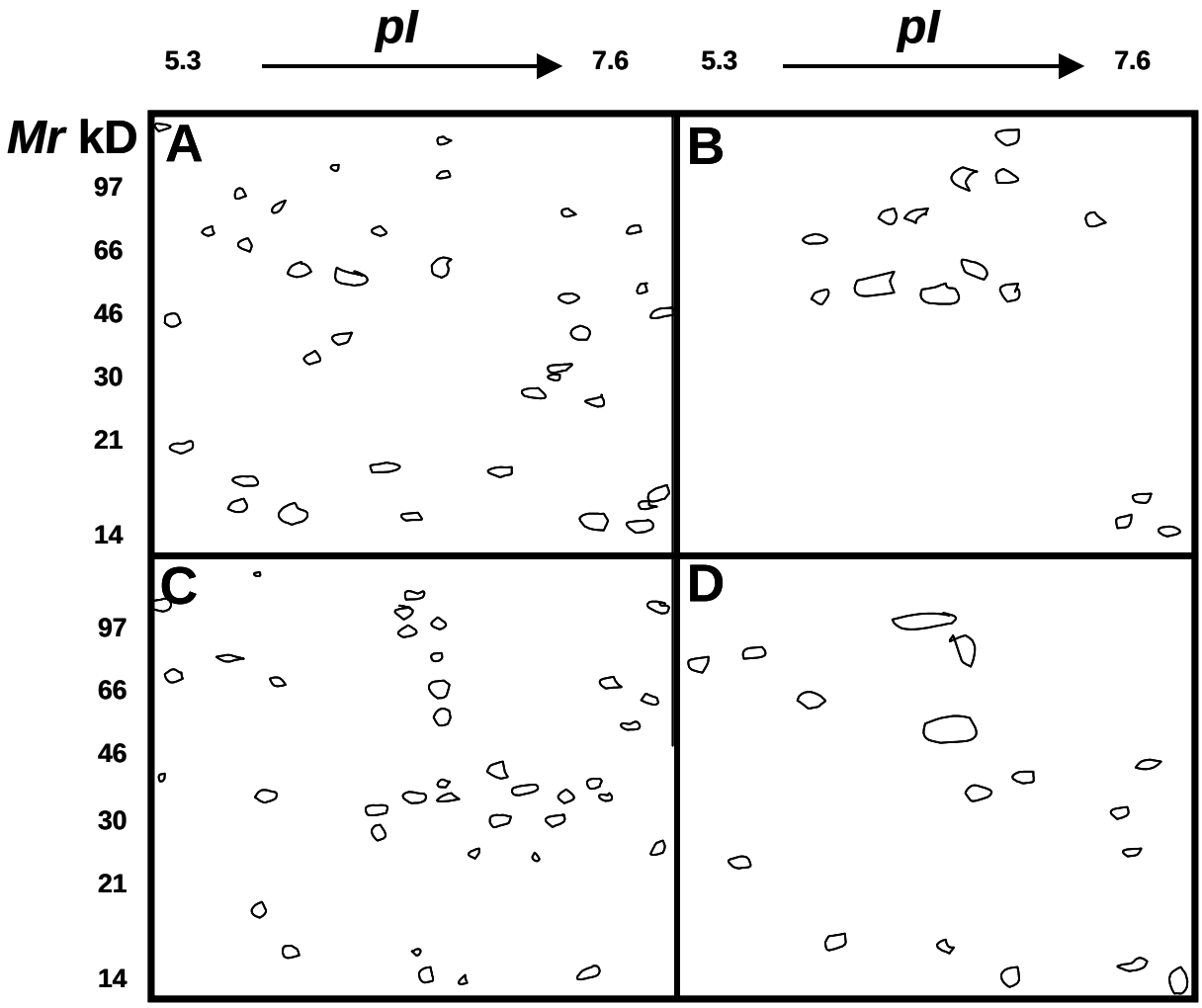

Fig. 3 : Cartoon representations of flourograms of two-dimensional gel electrophoresis of *de novo* synthesized proteins in mouse morulae (A,B) and blastocysts (C,D). The embryos were cultured with (B,D) and without (A,C) 50 ng/ml of TNF-α in M16 medium containing ^{35}S-methionine for 4 h.

proteins in lysates of morulae and blastocysts respectively after control (A) and 50 ng/ml of TNF-α (B) treatment. The matched Gausian images of flourograms of radioactive protein spots from control and TNF-α exposed embryos are represented by the cartoon image in Figure 3. Figure 4 shows the tiled images of differentially regulated protein spots, that are present in both control and TNF-α exposed embryos.

The computer aided matching analysis revealed a total of 35 spots in control morulae and 15 spots in TNF-α treated morulae, of which the intensity of the spots ranged from 23 to 118. Of these spots, 7 were matched representing 30.1% integrated optical density (IOD) and 43.3% IOD of total

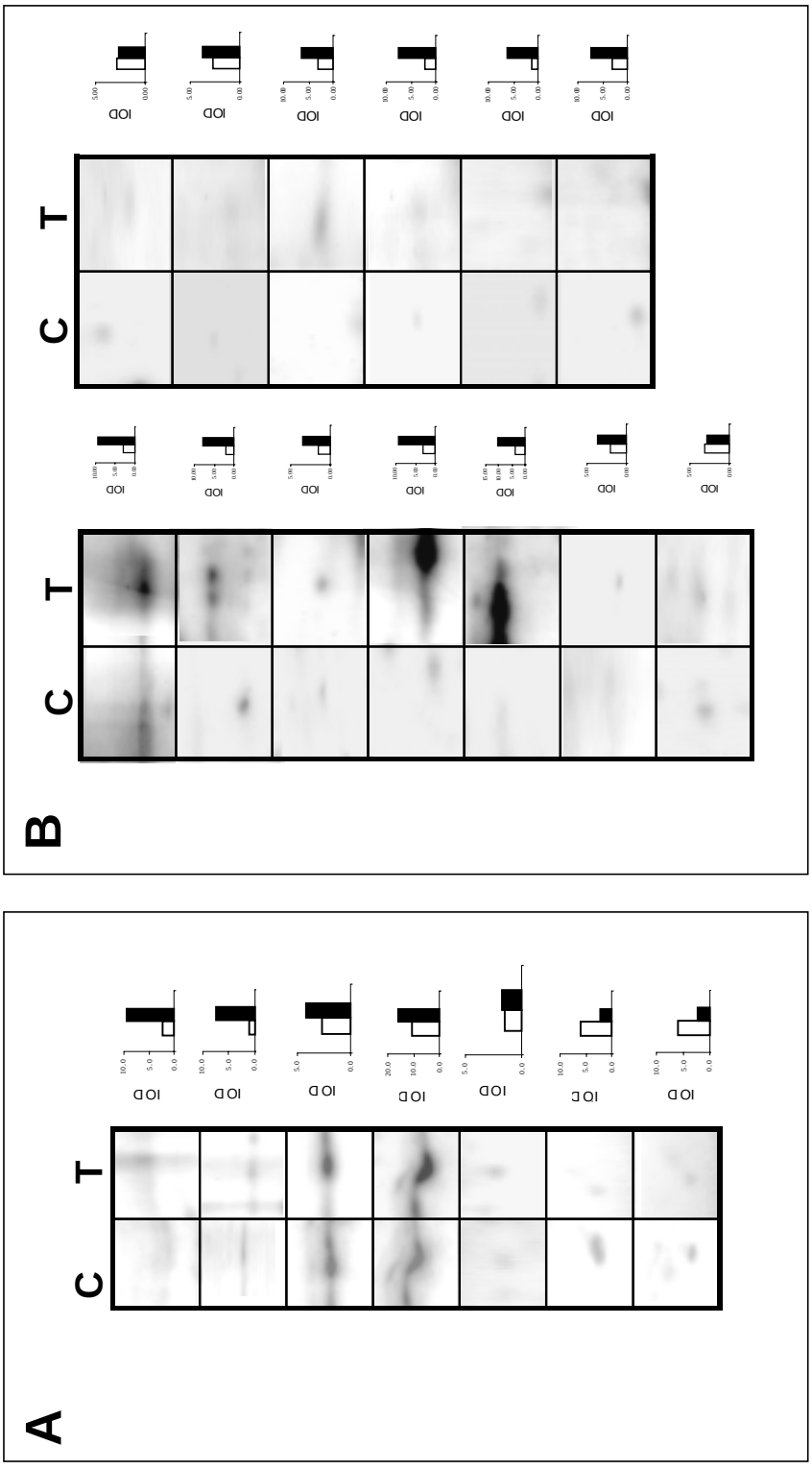

Fig. 4: Differentially expressed matched protein spots in control (C) and TNF-α (T) exposed morulae (A) and blastocysts (B) shown as tiled images along with their IODs. (C), control; (T), TNF-α.

staining density, respectively for control morulae and TNF-α (50 ng/ml) treated morulae (Fig. 4A). Thus, 28 spots representing 69.9% of optical density, which were observed in control group morula were not visible in fluorograms obtained from TNF-α treated morulae. 7 spots in TNF-α treated morulae represented 56.7% IOD exhibited different M*r* and *p*I vlaues as compared to those in control group. There were 8 new protein spots expressed only in morulae exposed to TNF-α. The 7 spots which were common in both control and TNF-α treated embryos are displayed as tiled images along with the bar diagram of their IOD (Fig. 5A). Out of the 7 common spots, the IOD of 3 spots were high in TNF-α treated morulae, while 2 spots showed a lower degree of expression.

A total of 40 spots in control and 17 in TNF-α exposed blastocysts were picked up by the software from the matched Gaussian images. The intensity of the faintest and the darkest spots were 19 and 157 respectively. Of these, 13 spots were common in control and TNF-α treated blastocysts. The IOD of the matched spots shown in the tiled images (Fig. 4B) represented 32.7% and 80.6%, of total staining density in control and TNF-α treated blastocysts. Thus, 27 spots representing 67.3% of optical density, which were observed in control group blastocysts were not visible in fluorograms obtained from TNF-α treated blastocysts. 4 spots in TNF-α treated blastocysts representing 19.4% IOD exhibited different M*r* and *p*I values as compared to those in the control group. Figure 5B shows the intensity distribution of matched points in terms of IOD%.

The protein map of the blastocyst lysate showed 28 new spots representing 74.2% of the total IOD as compared to morulae. 17 spots representing 68.8% of the total IOD which were seen in morulae were not detected in blastocysts. A protein spot of M*r* 95 kD and *p*I 6.7 not present in control morulae was seen in morulae treated with 50 ng/ml of TNF-α, as well as, in both groups of blastocysts.

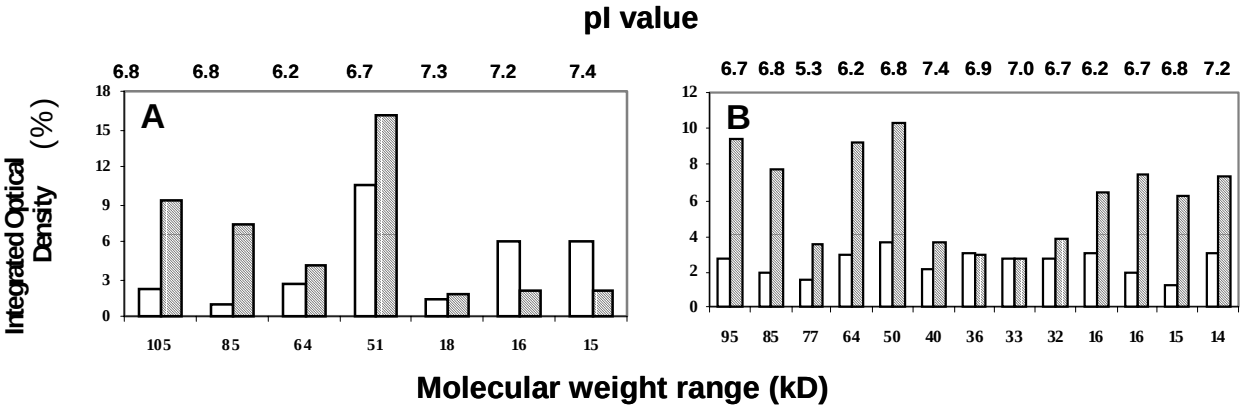

Fig. 5 : Histograms showing integrated optical densities (IOD) of matched protein spots synthesized by morulae (A) and blastocysts (B) as quantified from flourograms of two-dimensional electrophoresis. The embryos were exposed to either control medium (□) or 50 ng/ml of TNF-α (▨).

DISCUSSION

It is evident from the present study that TNF-α exposure *in vitro* adversely affects protein synthesis in morula stage and blastocyst stage preimplantation embryos. This observation corroborates well with the earlier *in vivo* and *in vitro* studies demonstrating significant reduction in the viability of mouse and rat blastocysts along with high level of apoptotic cell death following TNF-α exposure (11, 14, 15, 22). Also, there are reports indicating that the receptor for TNF (TNFR) are present on the surface of the preimplantation stage mouse and monkey embryos (23, 24). Thus the adverse effect of TNF-α observed in the protein synthetic ability of preimplantation stage embryo could be mediated through the TNFR present on preimplantation stage embryos.

In the present study, we observed that the exposure of morula and blastocyst to TNF-α alters protein synthesis by inhibition in total protein expression, as well as, decrease in the expression of specific proteins. Out of 15 spots observed in control morula, only 7 were detected in the flourograms of TNF-α treated morula, meaning 8 protein spots were missing in TNF-α treated morula. In blastocyst, out of 40 spots detected in control, only 13 could be seen in TNF-α treated embryos. Four specific proteins were detectable only in TNF-α exposed blastocysts, which may be acute phase proteins expressed in these embryos following the stress of cytotoxic action of TNF-α. Additionally, 8 and 17 proteins in control morulae and blastocysts were totally absent in their corresponding TNF-α exposed embryos. Thus, collectively it appears that the exposure of preimplantation stage embryos to TNF-α affects protein synthesis both quantitatively and qualitatively.

The method used in this study to map the protein expression pattern, the 2D electrophoresis is one of the most powerful and advanced analytical tool to study the functional proteomics in any biological system (25). Using this method, the proteins are separated based on their molecular weights and *p*I values, thus allowing for separation of a large number of proteins having same molecular weight. Although there is a report in the literature about the protein map of mouse embryos (26), this is the first report in which 2D electrophoresis has been used to study embryonic function in terms of protein synthesis. The present study limits itself to the analysis of number of quantitative and qualitative changes in the expression of protein. Further studies need to be done to characterize the differentially expressed proteins.

Finally, the results obtained from the present study corroborate well with our previous observation that increased levels of TNF-α in the uterine luminal content around the time of implantation following luteal phase treatment with potent antiprogestin like mifepristone adversely affect the developmental potential of preimplantation stage embryos (24).

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