

## Research Article



## Regional Variation in TUNEL and BCL-2 Expression in the Trophoblastic Tissue of Normal Human Placenta with Aging and Estimation of the True Apoptotic Index Ratio

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### ABSTRACT

This study aimed to evaluate the TUNEL and BCL-2 expression in syncytiotrophoblast and true apoptotic index ratio in preterm, term, and postdate placenta in different placental regions (cord insertion, central and peripheral) were done. A significant variation in the number of TUNEL stained positive cells at  $P \leq 0.05$  was evident among age groups in cord insertion and peripheral region of placenta, and a significant variations in the number of BCL-2 stained cells at  $P \leq 0.05$  among groups were evident in all regions in preterm placenta. The apoptotic index ratio was evidently increased with aging in all placental regions. A significant positive correlations was found between TUNEL stained positive cells and BCL-2 positive cells in postdate group in cord insertion region at  $r = 0.472$ , preterm group in central region at  $r = 0.601$ , and a significant negative correlation was found in postdate group in peripheral region at  $r = 0.57$ . These results indicated that TUNEL and BCL-2 showed various modes of expression in syncytium in different placental regions with aging, and the apoptotic index ratio represent the true measure of apoptosis, this emphasize the importance of apoptosis in permitting normal physiological turnover of villous trophoblast and also exhibits the contribution of BCL-2 in maintaining syncytial integrity throughout normal pregnancy.

**Keywords:** Apoptosis, BCL-2, Placenta, TUNEL.

### INTRODUCTION

The maintenance of structural and functional integrity of placental tissue involves a highly regulated cellular turnover that is dependent upon a delicate balance involving the cell proliferation, differentiation and death by apoptosis<sup>1</sup> The role of the BCL-2 protein is to extend cell survival by protecting the cell against apoptosis without affecting cell proliferation<sup>2,3</sup>, rescue of cells from apoptosis by BCL-2 can occur at any stage of the cell cycle.<sup>4</sup> Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) is a method used for detecting DNA fragmentation by labeling the terminal end of nucleic acids.<sup>5</sup>

### MATERIALS AND METHODS

A sample of 25 normal human placenta were collected from Al-Kadhimiya teaching hospital/ the obstetric and gynecological department, that delivered by C- sections, the mother age ranged from 18-35 years old, and gravidity ranged from G 1 -G3. The sample is divided into three groups: Preterm placenta (gestational age less than 37 weeks), term placenta (gestational age from 38 - 40 weeks), and postdate placenta (gestational age more than 40 weeks). Three different regions were selected from each age group, these regions were cord insertion region (at the base of umbilical cord), central region (at the center of the placenta), and peripheral region (2cm from placental margin). Tissues were fixed in 10% neutral buffered formalin, then proceeded through the steps of tissue preparation for paraffin blocks.<sup>6</sup> Sections were cut at (4 $\mu$ m) thickness serially started from maternal surface

of the placenta; placed on positively charged slides for TUNEL stain and immunohistochemistry for BCL-2. Monoclonal mouse anti-human BCL-2 Oncoprotein clone 124, code no. M0887 antibodies provided by DaKoCytomation, Denmark. Diluted in DAKO antibody diluent (S3022) was applied. Immunohistochemical stain for BCL- 2 was done, deparaffinization & rehydration, heat induced epitope retrieval in microwave –oven (MWO), Slides were incubated with peroxidase-blocking reagent. (K0679) for 15 minutes, then washed in TBS for 5 minutes, then incubated in primary antibody for 30 minutes, washed in TBS two times 5 minutes for each. Slides were incubated with biotinylated link antibody (K0679) for 20 minutes, then washed in TBS two times 5 minutes for each. Slides were incubated with Streptavidin/ Peroxidase (K0679) for 20 minutes, washed in TBS two times 5 minutes for each slides were incubated with DAB+(K0679) for 10 minutes (1 ml of DAB substrate is transferred into a small test tube and 1 drop of DAB chromogen is added and mixed. Slides were stained with counter stain (Mayer's Haematoxylin) (S3309). Evaluation of BCL-2 positive cells: by counting the number of BCL-2 positive cells in trophoblast layer of placental villi that exhibited intense, brown perinuclear staining.<sup>7</sup> TUNEL-based detection Kit provided by abcam (ab66108), that utilizes Terminal deoxyribonucleotidyl Transferase (TdT) to catalyze incorporation of fluorescein -12-dUTP at the free 3'-hydroxyl ends of the fragmented DNA. The fluorescein-labeled DNA can then be observed by the fluorescent microscope. Deparaffinization & rehydration, permeabilization of specimens: by diluting enough Proteinase K (STOCK: 2 mg/ml in 10 mM Tris pH 8) 1:100



was needed., equilibration and staining (50 µl) of staining solution was immediately applied onto each specimen, slides were placed in a humid chamber and incubated at 37°C for 1.5 hours, then were rinsed with 1X PBS.PI/RNase staining buffer diluted as 1:5 in PBS & applied 50 µl of diluted Propidium Iodide/RNaseA Solution onto the specimen. Incubate in the dark for 30 min at room temperature. Analyze the stain by fluorescence microscopy (apoptotic cells show bright yellow staining over an orange-red PI counter-staining). Statistical analysis of BCL-2 positive cells, and TUNEL positive cells in tissue sections was done by applying SSPS version 18. ANOVA-single factor analysis used to compare among the test groups in the selected regions (central, peripheral, and cord insertion) at  $P \leq 0.05$  that denoted as statistically significant. Tukey's test which is usually referred to as HSD (honestly significant difference) used as a multiple comparison test that make a use of a single value against which all differences in means are compared.<sup>8</sup> Correlation estimation between Tunel and BCL-2 was done and at  $P \leq 0.05$  that selected as the level of significance. True apoptotic index ratio: calculated as (number of Tunel positive cells/ number of BCL-2 positive cells)  $\times 100\%$ .

## RESULTS

Placental tissues that stained with Tunel for the apoptotic cells in syncytiotrophoblast overlying villi, revealed in cord insertion region the highest count of apoptotic cells were seen in preterm group  $28.3 \pm 10.5$ , with a significant variation in the number of apoptotic cells count among groups at  $p \leq 0.05$ , HSD: 33.66. Table (1), figure (1). In central region low count of apoptotic cells seen in postdate group  $19.7 \pm 5.9$ , analysis of variance among groups showed a non significant variation at  $p \leq 0.05$ , (HSD): 20.9 Table (1), figure (2). In peripheral region lowest count of apoptotic cells seen in postdate group  $14.4 \pm 4.9$ , analysis of variance among groups showed a significant variation at  $p \leq 0.05$ , HSD: 58.52 Table (1), figure (3).

**Table 1:** Mean  $\pm$  SD of apoptotic cells stained with Tunel in cord insertion, central and peripheral regions of preterm, term and postdate placenta.

Regions	Cord insertion	Central	Peripheral
Preterm	$28.3 \pm 10.5$	$19.5 \pm 10.1$	$19.7 \pm 5.3$
Term	$25.2 \pm 8.8$	$26 \pm 8$	$34.6 \pm 13.9$
Postdate	$16.8 \pm 6.7$	$19.7 \pm 5.9$	$14.4 \pm 4.9$
P value $\leq 0.05$	*0.02009 (significant)	0.15 (non significant)	*0.00000921 (significant)
HSD (Tukey's test)	33.66	20.91	58.52

Level of significance at  $P \leq 0.05$

BCL-2 was detected as a dense brown, cytoplasmic stain that was observed exclusively in the cytoplasm of the syncytiotrophoblast, and syncytial knots, it was enhanced over villi with subtrophoblastic fibrin deposition, while villi with perivillous fibrin deposition BCL-2 was absent or

discontinuous on their surface. In cord insertion region BCL-2 positive cells were seen predominantly in preterm group  $41.4 \pm 9.2$ , a significant difference was seen at  $p \leq 0.05$  & HSD: 85.32 (Table 2), figure (4). In central region BCL-2 positive cells were found predominantly in preterm group  $25.1 \pm 8.6$  with a significant difference among groups at  $p$ -value  $\leq 0.05$  with HSD: 59.1. (Table 2), figure (5). In peripheral region BCL-2 positive cells recorded mainly in preterm group  $18.3 \pm 7.9$ , and a significant variation among groups in BCL-2 expression at  $P$ -value  $\leq 0.05$  & HSD :27.16. (Table 2), figure (6).

**Table 2:** Mean  $\pm$  SD of Bcl2 positive cells and its significance in cord insertion, central and peripheral regions of the placenta in preterm, term and postdate groups.

Regions	Cord insertion	Central	Peripheral
Groups	mean $\pm$ SD	mean $\pm$ SD	mean $\pm$ SD
Preterm	$41.4 \pm 9.2$	$25.1 \pm 8.6$	$18.3 \pm 7.9$
Term	$22 \pm 5.5$	$9.4 \pm 3.4$	$9.2 \pm 5.3$
Postdate	$11.7 \pm 3.5$	$5.3 \pm 2.5$	$11.1 \pm 6$
p-value	*0.000000458	*0.0000000328	*0.011024
HSD	85.32	59.1	27.16

\*P value  $\leq 0.05$  considered statistically significant.

TUNEL/ BCL-2 ratio revealed an increase with placental maturation from preterm to postdate group in all placental regions. Table 3.

**Table 3:** TUNEL/ BCL-2 ratio in preterm, term, and postdate groups in different placental regions

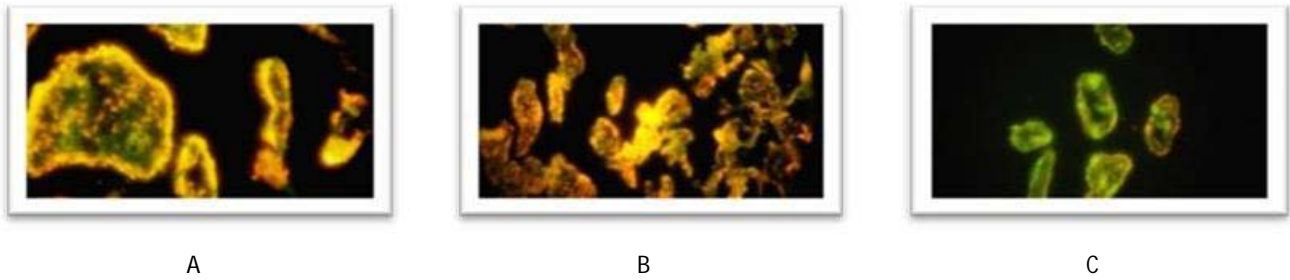
Regions	Preterm	Term	Postdate
Central	0.77	2.76	3.71
Peripheral	1.07	3.76	3.89
cord insertion	0.68	1.14	1.43

Regional estimation of correlation between TUNEL and BCL-2 in different placental regions revealed a significant positive correlation in preterm group in central region at  $r$ . 0.601 figure (7), and in postdate group in cord insertion region at  $r$ . 0.472, figure (8). A significant negative correlation was seen in postdate group in peripheral region at  $r$ . 0.57. figure (9) at  $p \leq 0.05$ .

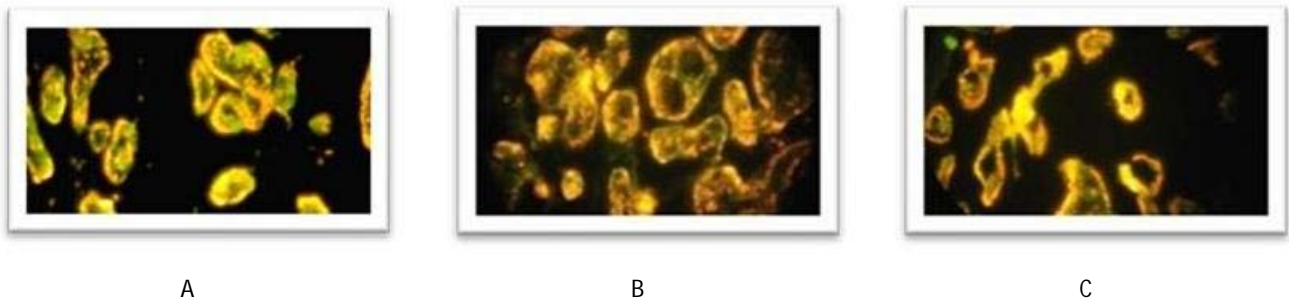
## DISCUSSION

In the present study apoptotic cells were seen within the villus is predominantly localized in the syncytiotrophoblast layer with virtually no events seen within cytotrophoblast. This has been mentioned that normally, apoptosis is triggered only when nuclei of trophoblast were in the syncytium<sup>9,10</sup> at the same time the syncytiotrophoblast demonstrated heterogeneous BCL-2 expression, this due to the fact that the apoptotic changes were not evenly distributed within the syncytium, being more advanced in some areas, especially at syncytial sprouts and knots, since BCL-2 is anti-apoptotic protein so we think this heterogeneity in apoptosis was also reflected on BCL-2 expression in syncytium.

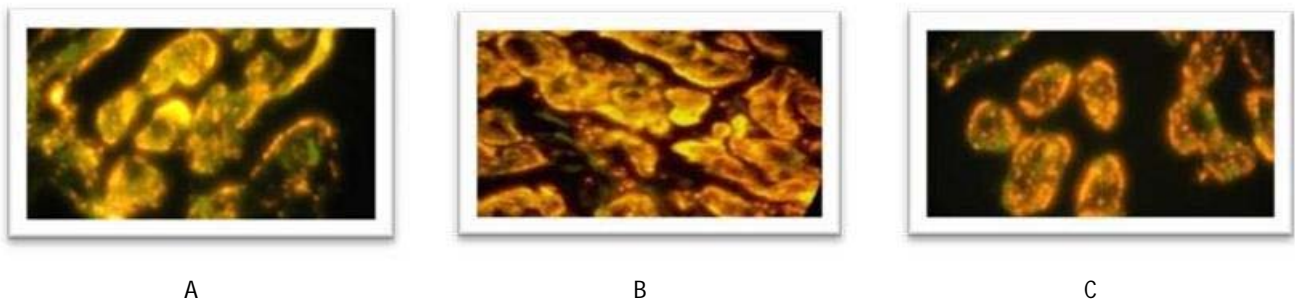




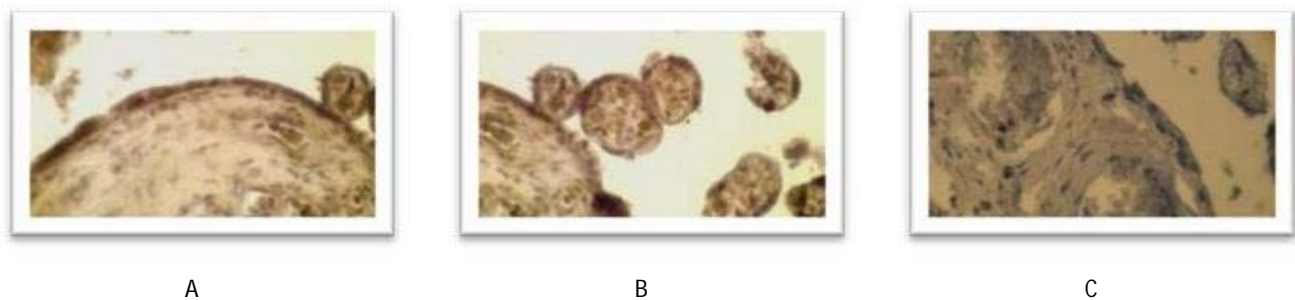
**Figure 1:** Cord insertion region of normal human placenta stained with TUNEL, in (A) preterm group, (B) term group, and (C) postdate group.40x



**Figure 2:** Central region of normal human placenta stained with TUNEL, in (A) preterm group, (B) term group, and (C) postdate group.40x



**Figure 3:** Peripheral region of normal human placenta stained with TUNEL, in (A) preterm group, (B) term group, and (C) postdate group.40x



**Figure 4:** Cord insertion region of normal human placenta stained with BCL-2, in (A) preterm group, (B) term group, and (C) postdate group.40x

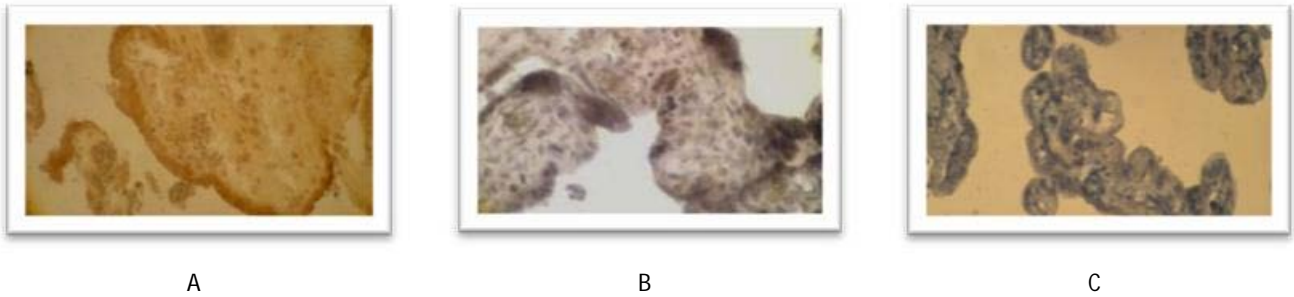
This could be due to that as long as fresh cytotrophoblast is incorporated into villous syncytiotrophoblast, syncytial apoptosis does not progress generally but rather only focally. These apoptotic foci in the syncytiotrophoblast are characterized by several features including focal loss of immuno reactivity for BCL-2, as it act as a force that

preventing DNA degradation from being spread to other nuclei in a multinuclear syncytiotrophoblast.<sup>11</sup>

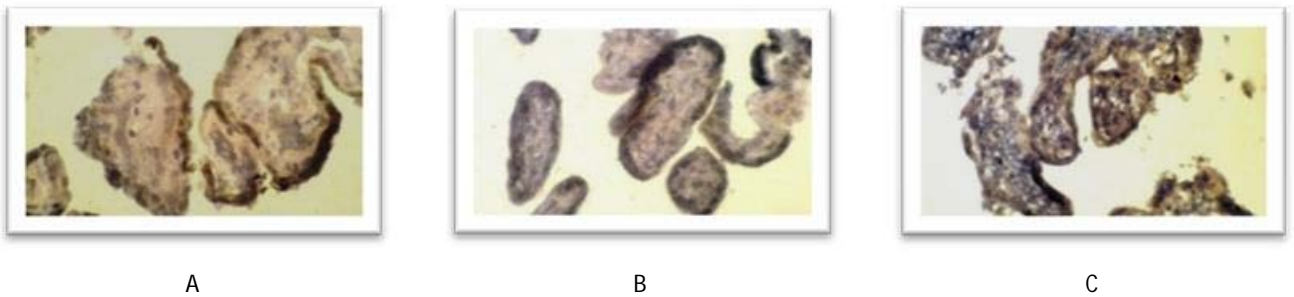
High values of BCL-2 positive cells that seen in preterm placenta of all regions, may be attributed to the protection of syncytium by BCL-2 against apoptotic force that preserve it to perform its actions in exchange and hormone production. This diffuse BCL-2 expression in

syncytiotrophoblast is difficult to understand, since syncytiotrophoblast has little or no ability to proliferate and the inhibitory effect on apoptosis exerted by BCL-2 generally leads to cell proliferation through immortalization of these cells.<sup>12,13</sup> Also BCL-2 protein was least abundant in term placenta compared to preterm placenta<sup>14</sup> and the degree of its expression was significantly decreased in the placenta after the gestational age of 32 weeks which coincides with the

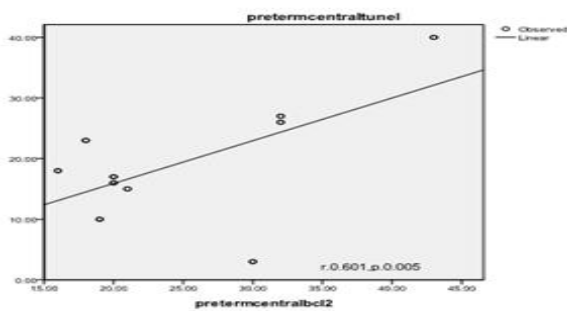
declining phase of placental growth, these results suggested two possible implications on the role of BCL-2 in placenta: it may be a type of proliferation or maturation-related marker, especially of trophoblast, which showed decrease expression along with terminal differentiation and maturation, and because the primary role of BCL-2 is the inhibition of programmed cell death (PCD), so decrease in placental BCL-2 around term may be a parturition-associated biological change.<sup>15</sup>



**Figure 5:** Central region of normal human placenta stained with BCL-2 in (A) preterm group, (B) term group, and (C) postdate group.40x

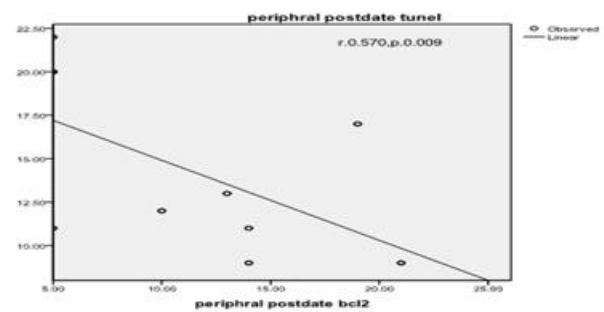


**Figure 6:** Peripheral region of normal human placenta stained with BCL-2 in (A) preterm group, (B) term group, and (C) postdate group.40x

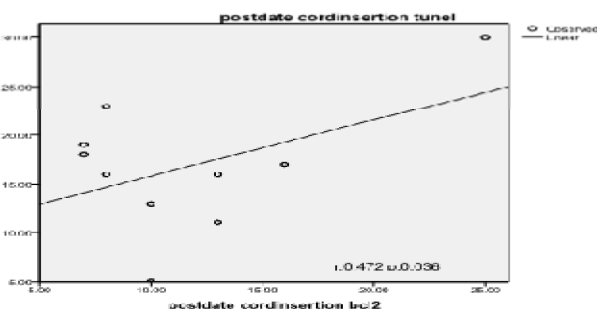


**Figure 7:** Positive correlation between TUNEL and BCL-2 in preterm group, central region. (Significant)

**Figure 8:** Positive correlation between TUNEL and BCL-2 in postdate group, cord insertion region. (Significant)



**Figure 9:** Negative correlation between TUNEL and BCL-2 in postdate group, peripheral region. (Significant)



A significant positive correlation that found between TUNEL stained cells and BCL-2 in preterm group in central region, and in postdate group in cordinsertion region, reflected that apoptosis in syncytiotrophoblast in these regions is opposed by high anti- apoptotic force of BCL-2, this since that BCL-2 is implicated in the regulation of cell death and survival without affecting cell proliferation, this may tend to preserve the syncytium.<sup>12,13</sup> While a

significant negative correlation that found between apoptosis and BCL-2 in postdate group in peripheral region, could suggested that apoptosis in postdate group is negatively influence BCL-2 expression, due to the effect of hypoxic cellular stress or oxidative stress, DNA damage, reactive oxygen species, or removal of growth factor support may lead to activation of the intrinsic pathway of apoptosis, and leads to alteration in mitochondrial membrane permeability due to an imbalance in the relationship of pro- and anti-apoptotic BCL-2 proteins.<sup>16</sup> The apoptotic index ratio increased with aging as long as BCL-2 expression decreased while Tunelstain increased in the syncytium. Furthermore during apoptosis, mitochondrial contents are released from the mitochondria, antagonizing anti-apoptotic inhibitor of apoptosis proteins.<sup>17,18</sup> Both extrinsic and intrinsic pathways of apoptosis culminate in a terminal pathway that involve the cleavage and activation of caspase-3, 6, and 7 initiating cell destruction by activating DNases and cleaving DNA repair enzymes.<sup>19, 20</sup>

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