# CHAPTER IV

# RESULTS AND DISCUSSION

**A functional impact of CD95 on the induction of eryptosis**

In order to study the pro-eryptos is function of death receptor CD95 in red blood cells. The normal human red blood cells were treated with the agonistic CD95 antibody CH11 at various concentrations at 0, 100, 300 and 500 ng/ml for 24, 48 and 72 hours that can increase eryptosis in a dose dependent manner in nucleated cells was perform as described in Methods. The functional detection of CD95 for induction of eryptosis in red blood cells, phosphatidylserine exposure measurement on the outer surface membrane, that is critical for the efficient clearance of apoptotic cells by flow cytometry after staining the red blood cells with FITC-labeled Annexin V. The data showed that percentage eryptotic cells in red blood cells with CH 11 at concentrations 0, 100, 300 and 500ng/ml for 24, 48 and 72 hours were not significant and are shown in Table 3 and Figure 7

In positive control of activity CH11for stimulation of apoptosis in Jurkat cells, the percentage of apoptotic cells were 51% compared to 13% in an untreated sample (Figure 8) and positive control for red blood cells apoptosis (eryptosis), treated red blood cells with ionomycin for 2 hours the percentage of apoptotic cells were 100% (Figure 8)

**Table 3 The effect of CH11 on the function of death receptor CD95 red blood cells**

| sample | % apoptotic cell | | |
|---|---|---|---|
| | 24 h | 48 h | 72 h |
| RBC with 0 ng/ml CH11antibody | 3 | 4 | 24 |
| RBC with 100 ng/ml CH11antibody | 5 | 5 | - |
| RBC with 300 ng/ml CH11antibody | 4 | 5 | - |
| RBC with 500 ng/ml CH11antibody | 4 | 5 | 28 |

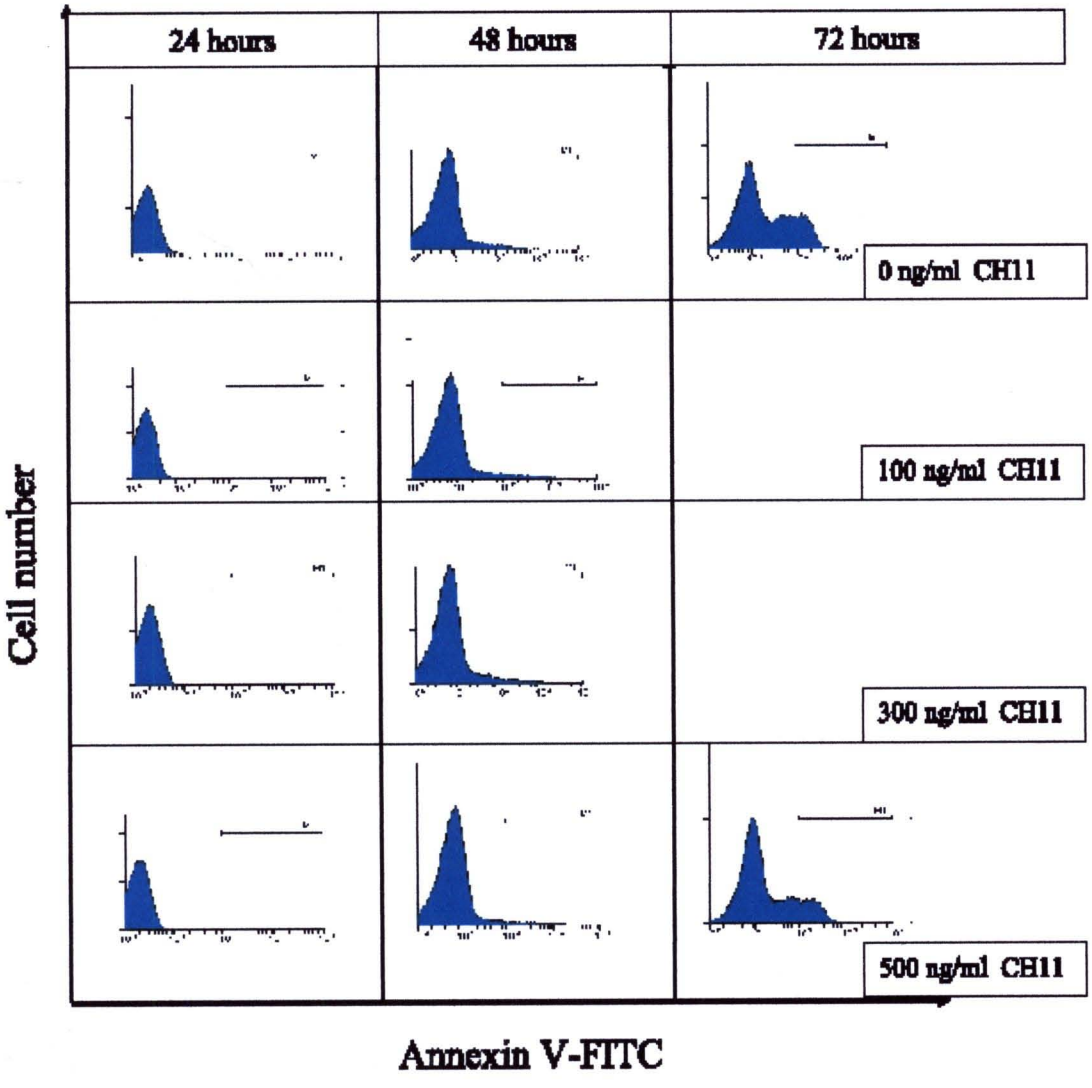


**Figure 7 Histrogram of the effect of CH11 on the function of death receptor CD95 red blood cells. Red blood cells were treated with increasing concentrations of CH11 to stimulation of eryptotic cells**

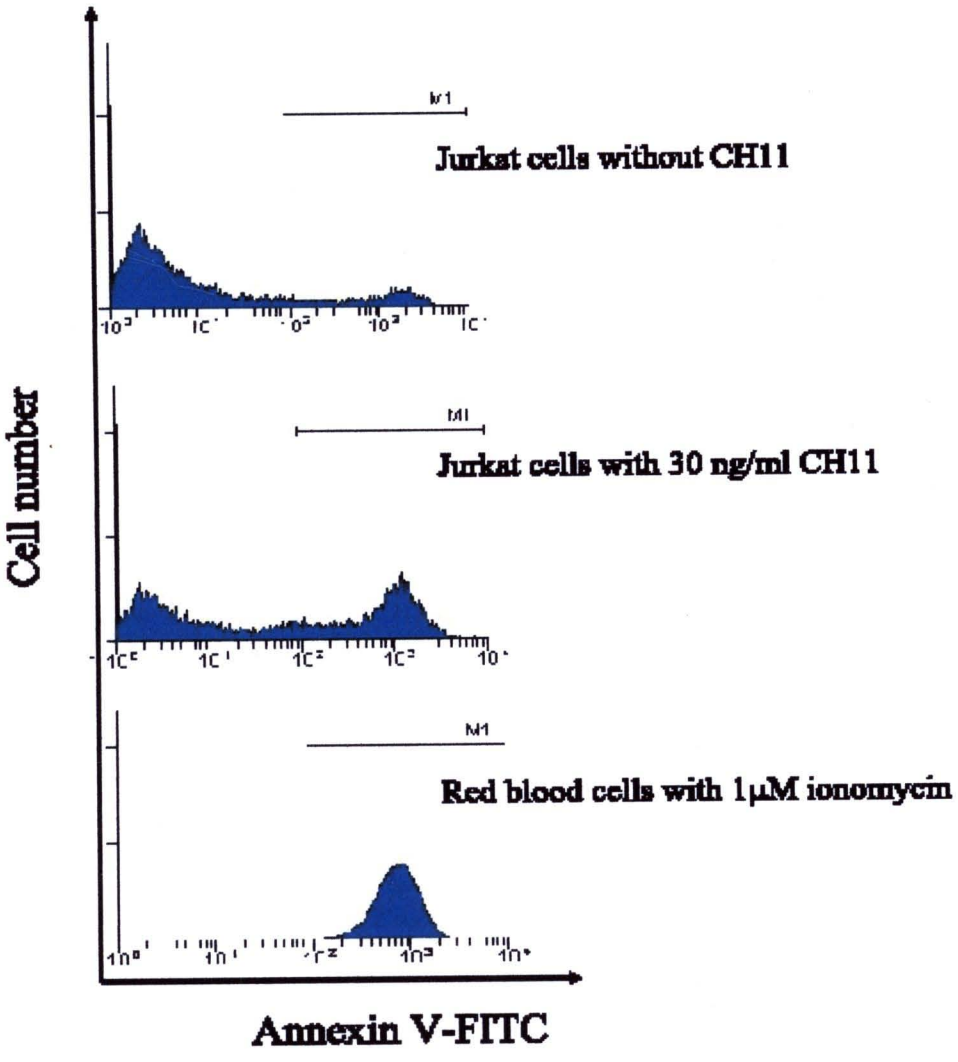


**Figure 8 Positive control of activity CH11for stimulation of apoptosis in Jurkat Cells and red blood cells with ionomycin**

**Influence of insulin to reduce eryptosis**

1. **Effect of insulin concentrations and time of glycolysis in red blood cells**

Induction of glycolysis in red blood cells by addition of insulin various concentrations at 0, 10, 100 and 1,000 mM for 1, 2, 3 and 4 hours. After incubation, measure lactate concentration (product of glycolysis) by chemistry analyzer (Hitachi 912, Roche). The result showed that the concentrations of lactate were slightly increased with the increasing time of incubation. In set of experiments shown that treatment of glycolysis in red blood cells for 3 hours with 10 mM insulin induced

highest lactate (Table 4). The duplicate result of insulin concentration at 10 mM for 3 hours incubation time was optimal of red blood cells glycolysis (Table 5).

**Table 4 Lactate concentration was determined using the automated chemistry analyzer by Hitachi 912, Roche**

| samples treated by insulin | Lactate concentration (mg/dl) | | | |
|---|---|---|---|---|
| | **1 h** | **2 h** | **3 h** | **4 h** |
| RBC with 0 mM insulin | 0.16 | 0.16 | 0.22 | 0.32 |
| RBC with 10 mM insulin | 0.22 | 0.25 | 0.41 | 0.47 |
| RBC with 100 mM insulin | 0.25 | 0.25 | 0.35 | 0.22 |
| RBC with 1,000 mM insulin | 0.25 | 0.22 | 0.35 | 0.25 |

**Table 5 Lactate concentration with 10 mM insulin in red blood cells**

| samples treated by 10 mM insulin | Lactate concentration (mg/dl) | | | | |
|---|---|---|---|---|---|
| | **0 h** | **1 h** | **2 h** | **3 h** | **4 h** |
| Sample 1 | 0.16 | 0.47 | 0.72 | 0.75 | 1.12 |
| Sample 2 | 0.32 | 0.38 | 0.81 | 0.91 | 1.12 |

**Effects of the red blood cells with tBOOH and $H_2O_2$ to determine a suitable concentration range for induction of red blood cells eryptosis**

Inductions of oxidative stress by additions of tBOOH and $H_2O_2$ various concentrations at 0, 0.33, 0.66, 0.1 mM tBOOH and 0.0, 1.0, 2.0, 4.0mM $H_2O_2$ for 24 hours. After incubation, phosphatidylserine exposure measurement on the outer surface membrane, that is critical for the efficient clearance of apoptotic cells by flow cytometry after staining the red blood cells with FITC-labeled Annexin V. The result showed that the concentrations of tBOOH at ≤ 0.33 mM were induced to phosphatidylserine exposure in red blood cells (Figure 9). $H_2O_2$ don't induce to phosphatidylserine exposure in red blood cells.

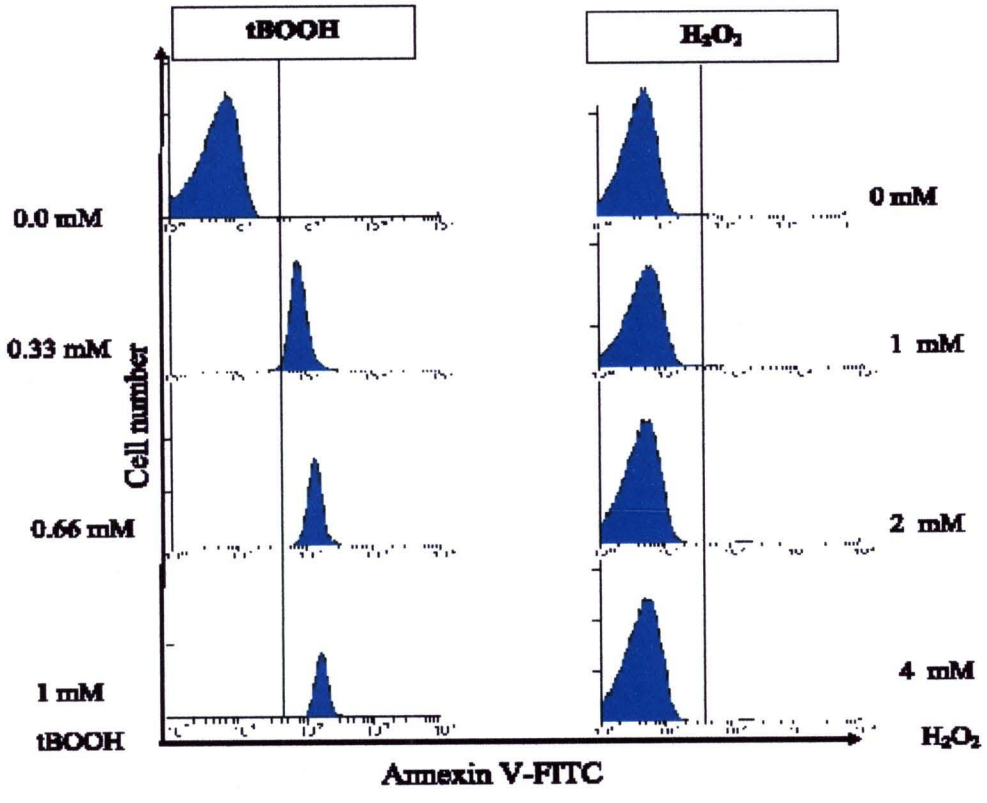


**Figure 9 Histrogram of effects of the red blood cells with tBOOH and $H_2O_2$ to determine a suitable concentration range for induction of red blood cells eryptosis**

Red blood cells were treated with increasing concentrations of tBOOH that they were viable undergoing eryptosis or annexin V-FITC positive and not undergoing eryptosis or annexin V-FITC negative treated with $H_2O_2$.

**Investigation of eryptotic cells on oxidative stress of normal and thalassemic red blood cells**

Previous experiment have shown effect of insulin concentrations at 10 mM for 3 hours incubation time was optimal of glycolysis in red blood cells and effects of the red blood cells with tBOOH at 0.33 - 0.1 mM was optimal of oxidative stress inductance. Demonstration of the PS positive on the exofacial surface of both normal and thalassemic RBCs in oxidative stress with insulin was perform as described in methods and that stained with annexin V-FITC and analyzed by FACSort flow cytometer.

The data shown eryptotic cells of normal and thalassemic RBCs were shown in Table 6. Values were expressed as percentage of eryptotic cells. Eryptotic cells of normal RBCs in oxidative stress treated by 0.02mM tBOOH absence and presence insulin in experiment number 2 decreased from 66% to 42%, there were some experiment with 0.02mM tBOOH did not induce eryptotic cells (such as exp# 1 and 3)

Eryptotic cells of normal RBCs in oxidative stress treated by 0.1mM tBOOH absence and presence insulin in experiment number 1-3 decreased from 55% to 46%, 90% to 58% and 72% to 54%, respectively (Figure 11).

Eryptotic cells of Beta thalassemia/HbE RBCs in oxidative stress treated by 0.02mM tBOOH absence and presence insulin in experiment number 2 and 3 decreased from 66% to 41% and 77% to 48% respectively, but experiment 1 with 0.02mM tBOOH did not induce eryptotic cells. Eryptotic cells of Beta thalassemia/HbE RBCs in oxidative stress treated by 0.1mM tBOOH absence and presence insulin in experiment number 1-3 decreased from 73% to 7%, 97% to 79% and 97% to 95%, respectively (Figure 12).

Eryptotic cells of Hb H disease RBCs in oxidative stress treated by 0.02mM tBOOH absence and presence insulin in experiment number 3 decreased from 31% to 17%, there were some experiment with 0.02mM tBOOH did not induce eryptotic cells

(such as exp# 1 and 2). Eryptotic cells of Hb H disease RBCs in oxidative stress treated by 0.1mM tBOOH absence and presence insulin in experiment number 1-3 decreased from 81% to 22%, 70% to 51% and 80% to 57%, respectively (Figure 13).

**Table 6 Percentage of eryptotic cells various concentration of tBOOH. The data demonstrated the percentage of eryptotic cells from three experiment of Normal, Beta thalassemia/HbE and Hb H disease red blood cells**

| Sample | tBOOH concentration (mM) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 0 mM | | | 0.02 mM | | | 0.1 mM | | |
| | Eryptotic cells (%) | | | Eryptotic cells (%) | | | Eryptotic cells (%) | | |
| | Exp# 1 | Exp# 2 | Exp# 3 | Exp# 1 | Exp# 2 | Exp# 3 | Exp# 1 | Exp# 2 | Exp# 3 |
| Normal RBCs | | | | | | | | | |
| without | 1 | 1 | 1 | 1 | 66 | 10 | 55 | 90 | 72 |
| insulin | 1 | 1 | 1 | 1 | 42 | 4 | 46 | 58 | 54 |
| with insulin | | | | | | | | | |
| Beta/HbE | | | | | | | | | |
| RBCs | 1 | 3 | 26 | 1 | 66 | 77 | 73 | 97 | 97 |
| without | 1 | 3 | 18 | 1 | 41 | 48 | 7 | 79 | 95 |
| insulin | | | | | | | | | |
| with insulin | | | | | | | | | |
| Hb H RBCs | | | | | | | | | |
| without | 1 | 1 | 2 | 1 | 2 | 31 | 81 | 70 | 80 |
| insulin | 1 | 1 | 2 | 3 | 1 | 17 | 22 | 51 | 57 |
| with insulin | | | | | | | | | |

**Note:** Exp# = experiment number

The data shown eryptotic cells of normal and thalassemic RBCs were shown in Table 7. Values were mean ± S.E. and expressed as percentage of eryptotic cells. Eryptotic cells of normal RBCs group in oxidative stress absence and presence insulin decreased from 49.00 ± 14.56% to 34.16 ± 10.28% (P =0.029). In thalassemic RBCs group, eryptotic cells were decreased from 56.33 ± 10.72% to 35.00 ± 9.22% (P=0.006).

The data shown that the percentage of eryptotic cells in oxidative stress with insulin were significantly different from without insulin (Figure 10)

**Table 7 Mean ± S.E. of percentage of normal eryptotic cells of normal and thalassemic group in oxidative stress**

| Sample | Oxidative strees ( tBOOH 0.02 mM) | | Oxidative strees ( tBOOH 0.1 mM) | |
|---|---|---|---|---|
| | without insulin | without insulin | with insulin | with insulin |
| | Eryptotic cells (%) | Eryptotic cells (%) | Eryptotic cells (%) | Eryptotic cells (%) |
| Normal RBCs group | 49.00 ± 14.56 | 49.00 ± 14.56 | 34.16 ± 10.28* | 34.16 ± 10.28* |
| Thalassemic RBCs group | 56.33 ± 10.72 | 56.33 ± 10.72 | 35.00 ± 9.22* | 35.00 ± 9.22** |

**Note:** * P =0.029, ** P=0.006 significantly different when compared with untreated group

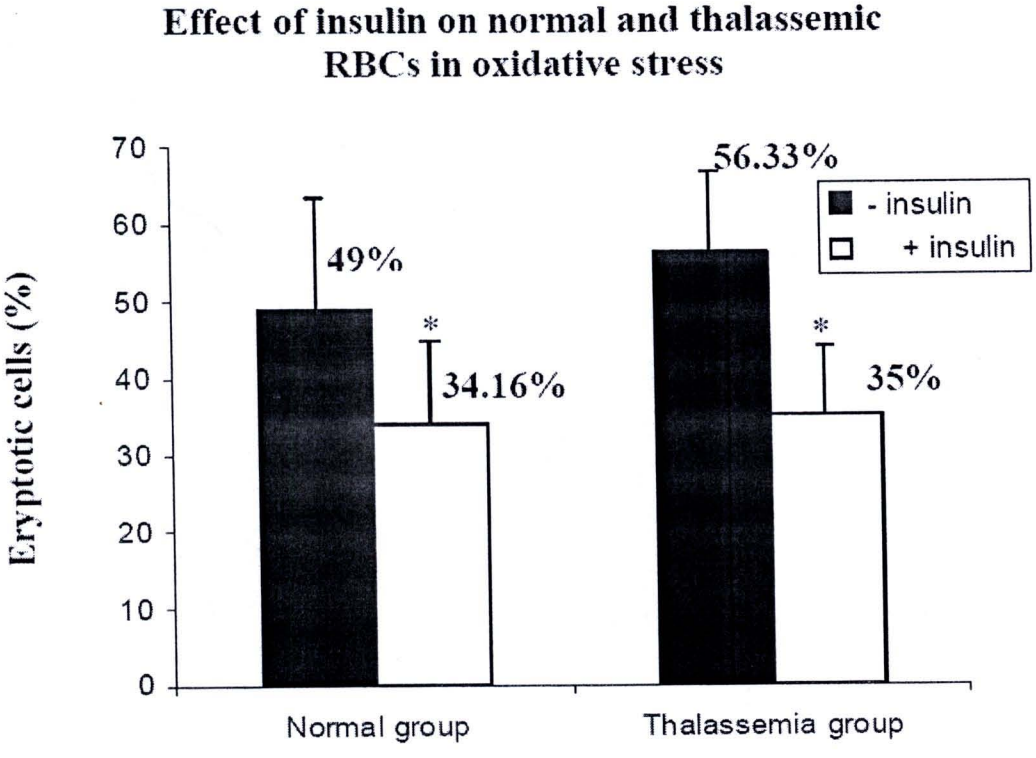


**Figure 10 Eryptotic cells of normal and thalassemic group in oxidative stress absence and presence insulin**

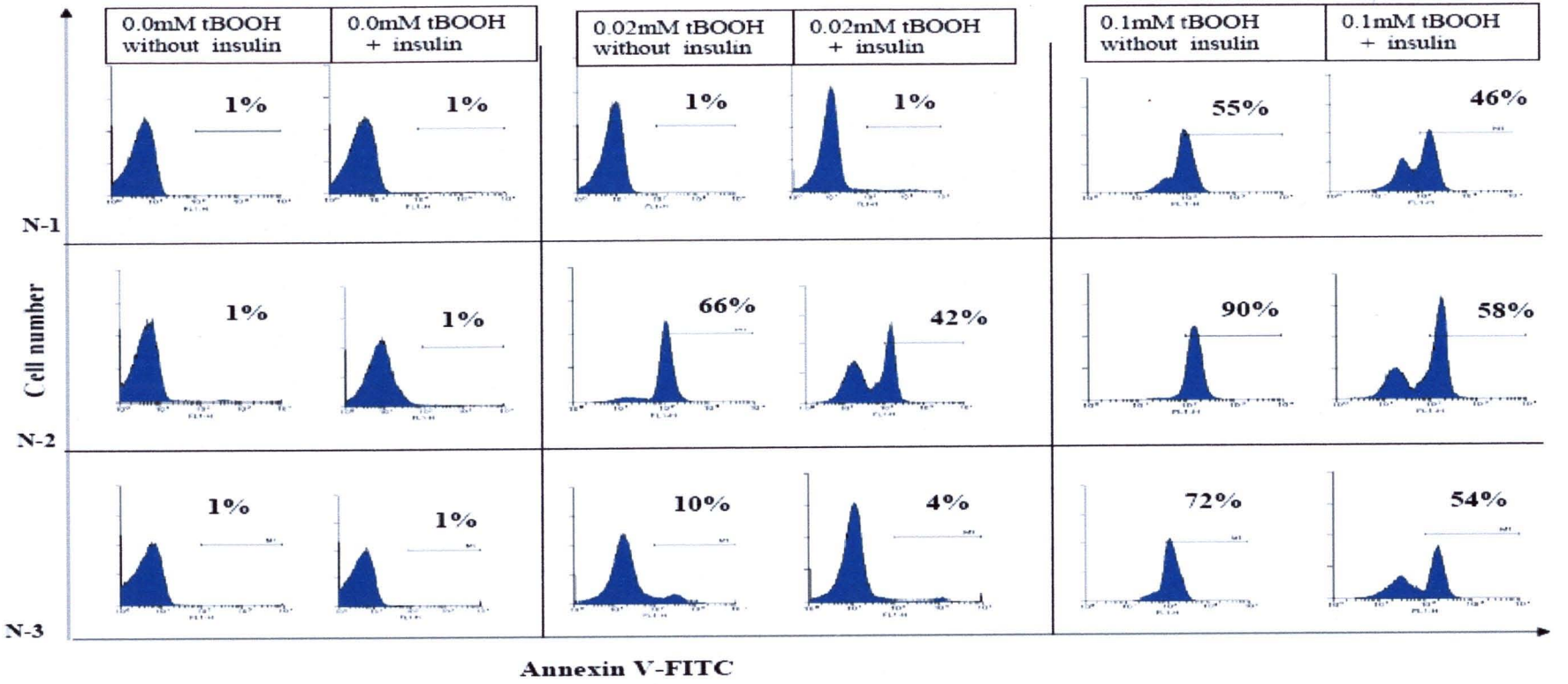


**Figure 11 Histrogram of the percentage at each conditions of PS positive normal red blood cells in oxidative stress**

**Note:** N-1 = normal red blood cell sample 1, N-2 = normal red blood cell sample 2, N-3- = normal red blood cell sample 3

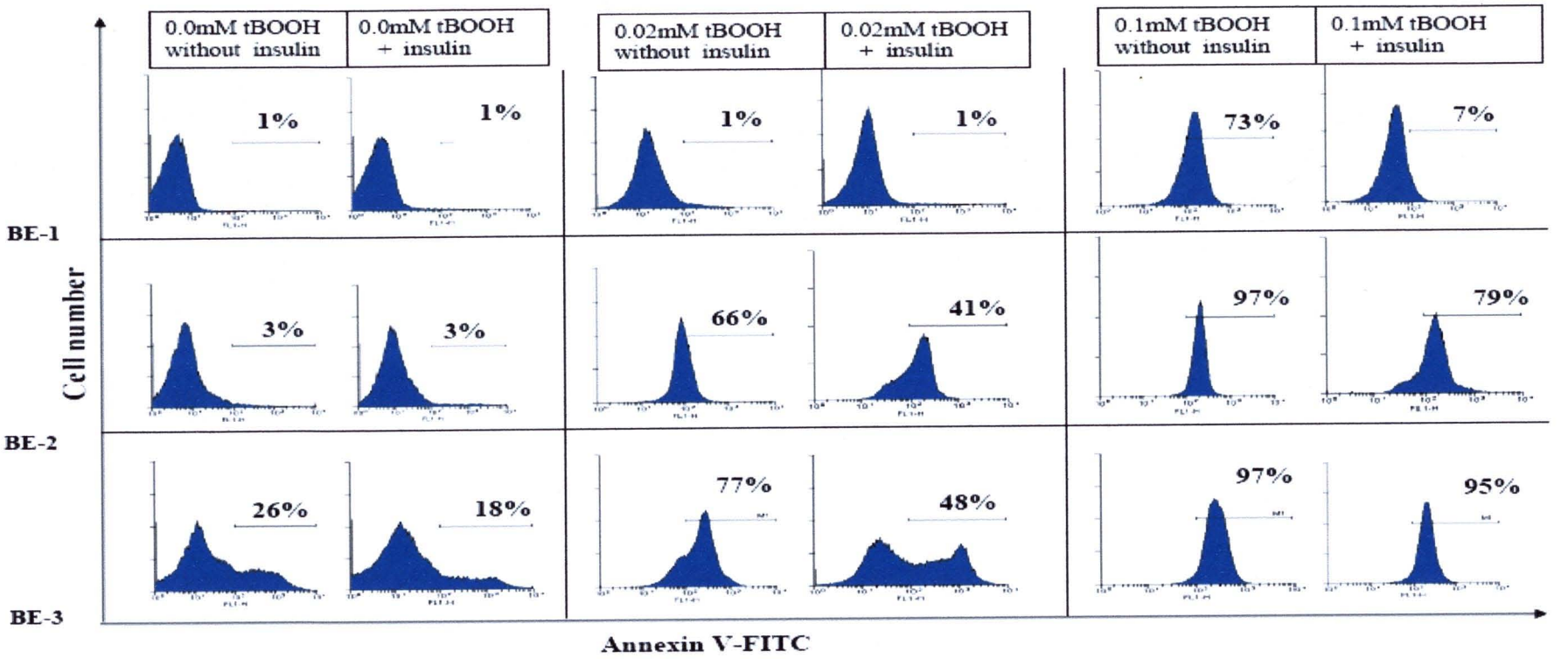


**Figure 12 Histrogram of the percentage at each condition of PS positive Betathal/HbE red blood cells in oxidative stress**

**Note:** BE-1 = Beta thal/HbE red blood cell sample 1, BE-2 = Beta thal/Hb E l red blood cell sample 2,

BE-3- = Beta thal/Hb E red blood cell sample 3

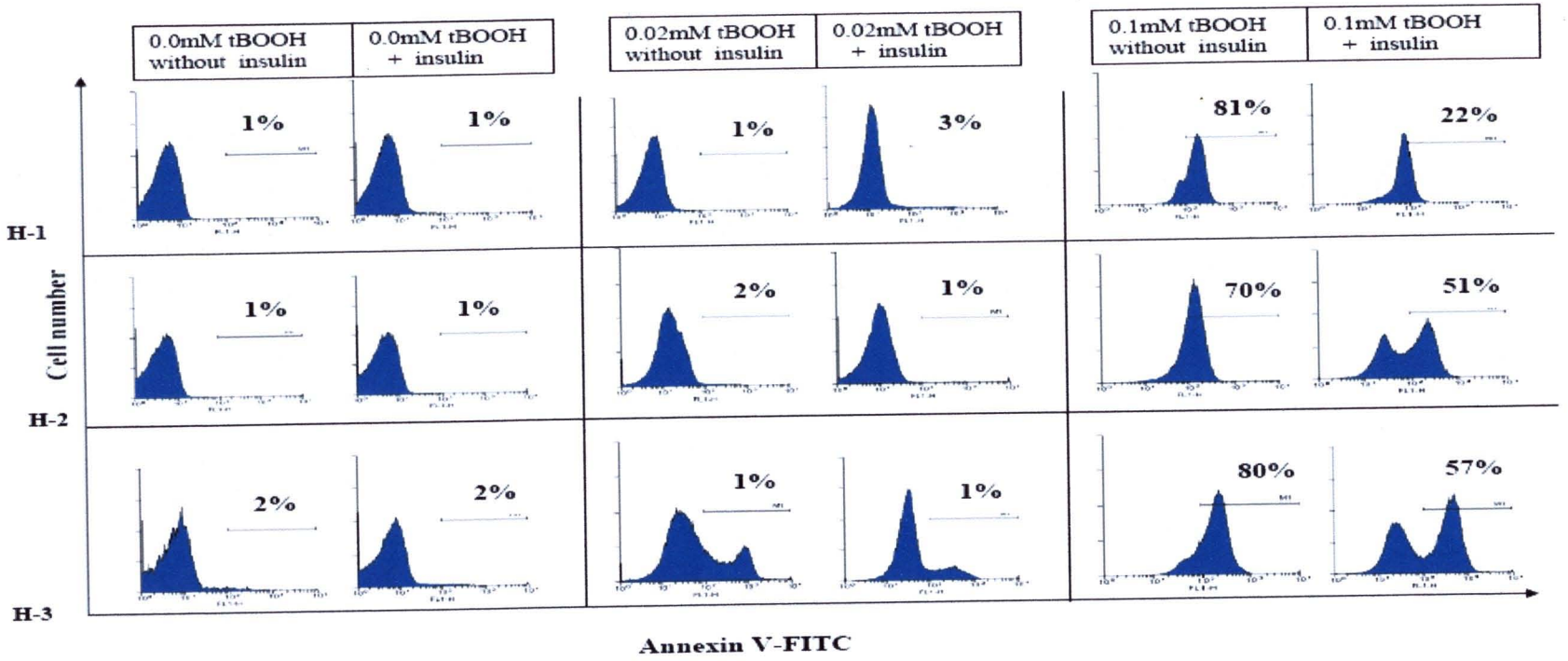


**Figure 13 Histrogram of the percentage at each conditions of PS positive Hb H red blood cells in oxidative stress**

**Note:** H-1 = Hb H red blood cell sample 1, H-2 = Hb H red blood cell sample 2, H-3- = Hb H red blood cell sample 3