

IN VITRO BIOLOGICAL ACTIVITY ASSESSMENT OF RHEPO PHARMACEUTICAL FORMULATIONS FOR BATCH RELEASE

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ABSTRACT

Background: Recombinant human erythropoietin (rhEPO) is the pharmaceutical form of erythropoietin (EPO), used for the treatment of chronic kidney disease and cancer-associated anemia, act as a growth proliferation factor for cells having EPO- receptor on their surfaces such as blood progenitors, non-hematopoietic cells and some types of tumor cells. **Methodology:** This study is objectively planned for *in vitro* biological assessment of rhEPO activity as a routine quality control testing, instead of *in vivo* animal testing. Malignant cell lines such as MCF-7, HepG2, HELA and PC3 were subjected as experimental cells in this study. Growth rate response for rhEPO treatment was estimated by MTT- based bioassay then was confirmed by hemacytometer and microscopical observation. **Results:** Statistical records reflected that rhEPO at concentration of 0.78 IU/ml to 25 IU/ml causes cell proliferation exponentially at $r > 0.9$. Using single factor ANOVA (T-test), there is a significant cell proliferation at $p < 0.005$ comparing with non-treated cells. **Conclusion:** Cell proliferation corresponding to rhEPO can be used as a new approach for quality control testing of rhEPO pharmaceutical products.

KEYWORDS: hemacytometer and microscopical.

1. INTRODUCTION

Erythropoietin (EPO) was successfully developed in the 1980s and considered as the primary modulator of red blood cell production. It has been widely used in the treatment of anemia caused by chronic kidney disease, blood loss and chemo-radiotherapy induced myelodysplasia.^[1]

Several studies have identified the presence of EpoR in normal cells and tumor cells^[2], the expression of EpoR by tumor cells has been emphasized via immunohistochemical and immunochemical methods^[3],^[4] and other *in vitro* studies have showed that Epo causes tumor proliferation.^{[5], [6], [3]}

Epo binding induces the cascade stimulation of Jak2 tyrosine kinase leading to activation of different intracellular pathways as Ras/MAP kinase, phosphatidylinositol 3-kinase and STAT transcription factors resulting in cell proliferation and differentiation.^{[7], [8], [9], [10], [11], [6]} Negative control of that signal transduction cascade is mediated through ubiquitination of EpoR on the cell surface and subsequent proteosomal degradation.^[12] This mechanism are well

reported in erythroid cells, but has not been reported in tumor cells.^[13]

The huge market demand for rHuEPO products requires more accurate and convenient *in vitro* assay as an alternative to current methods including animal test^[14], immunological assays^[1] and *in vitro* method using erythroid cells colony formation to facilitate the rapid batch release of rHuEPO formulations.^{[1], [15]}

EPO responsive tumor cell lines expressing functional EPO-R could be used to assess the bioactivity of rhEpo formulations.^{[16], [15], [17]}

2. MATERIALS AND METHODS

2.1 Reagents

All reagents are within their shelf life, WHO International Standard Erythropoietin, Human recombinant code no.88/574 (NIBSC)^[18], RPMI1640w/phenol red (Biowest), fetal bovine serum (Biowest), Trypsin 0.05%, EDTA (Euo-clone) and MTT dye (HIMEDIA). All these reagents are kindly supplied by National Organization for Research and Control of Biologicals NORCB.

2.2 Cell lines

The cancer cell lines demonstrated in the following table were supplied to the Egyptian holding company for vaccines and Sera (VACSERA) via the American Type Culture Collection (ATCC; Manassas, VA, USA).

Table 1: List of cell lines used in the proliferation assay.

Type	Name	ATCC no.
Cell lines	MCF-7	ATCC HTB-22™
	HepG 2	ATCC HB-8056™
	PC- 3	ATCC CRL-1435™
	Hela	ATCC CRM-CCL-2™

2.3 Cell line maintenance

Cell lines were cultured according to the recommended instructions in basic growth medium, RPMI 1640 supplemented with 10% fetal bovine serum (FBS) at 37°C, the cell lines viability were examined microscopically and ensure that they were free from any contaminations and assess degree of confluence before proceeding proliferation test.

2.4 Proliferation assay

Each cell line is plated in triplicates into 96-well plates at a concentration of 5×10^3 cells per well in growth medium RPMI 1640 with 0.1% FBS. After 24 h, cells are subjected to serum starvation for 24 h, using growth media free serum. Each group was treated with different dilutions started from 50 IU/ml of EPO NIBSC standard to 0.78 IU/ml in addition to control untreated cells. Thereafter, the cultures were incubated at 37°C for 3 days, cells were observed daily. sulforhodamine B (SRB) staining was used 3 days post treatment for microscopically observation (fig. 5).

Cell proliferation was quantified using (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay. MTT is a pale yellow substrate, which is reduced to a dark blue insoluble formazan product, when incubated with living cells. The amount of formazan uptake is measured by 96 well plate ELISA reader at 630 nm. The MTT results for different cell lines were confirmed by Cell Counting with Neubauer Chamber hemacytometer using viability staining Typan blue fig. 3.

3. RESULT

The rhEPO bioactivity was assessed based on tumor cell proliferation. Dose –response curves for each cell line were plotted followed by determination of (r) and calculation of cell proliferation. Data analysis was carried out using (General liner model, regression analysis). Statistic variables such as arithmetic mean, standard deviation (SD), coefficient of variance (CV) describing the precision of the analytical method, recovery % (R%) describing the accuracy of the analytical method and correlation factor (r) are used. A high correlation coefficient is often recommended as evidence of good fitness. The statistical requirements for

assay validity is a significant regression such that $r \geq 0.9$ and $CV \leq 15\%$ according to international conference of harmonization (ICH) guideline www.ich.org/products/guidelines/quality.htm.

Cell proliferation was found to be directly proportional to the ascending concentrations of rhEPO. When cells were reached exponential growth, the accumulation of formazan in control non-treated cells was significantly lower than proliferating cells cultured in growth medium containing EPO as shown in fig.1 and 2.

The CV between replicate counts, expressed as within-run variation, was not more than 15%, the correlation factor (r) > 0.9 and proliferation was significant at $p < 0.005$ when comparing EPO treated cells with non-treated cells using single factor ANOVA (T-test).

The bioactivity of rhEPO pharmaceutical formulation e.g. Eprex can be assessed against rhEPO international standard such that both rhEPO test sample and standard are tested under the same conditions using the same cell line as shown in fig.4.

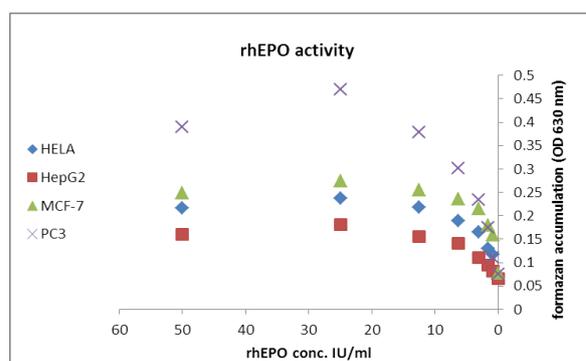


Figure 1: rhEPO bioassay using Hela, HepG2, MCF-7 and PC3 cell lines treated with increasing concentrations of EPO as indicated. Cell proliferation was assessed at 72 hr using the MTT assay at 630 nm. Each point represents the average of three independent determinations.

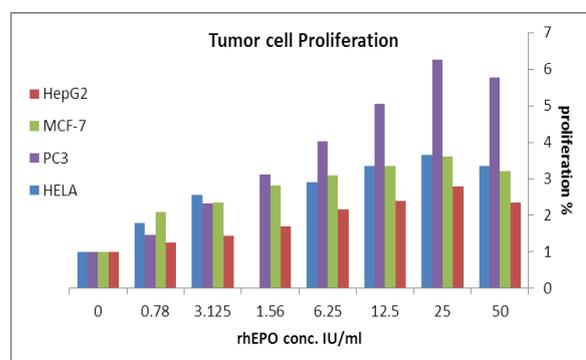
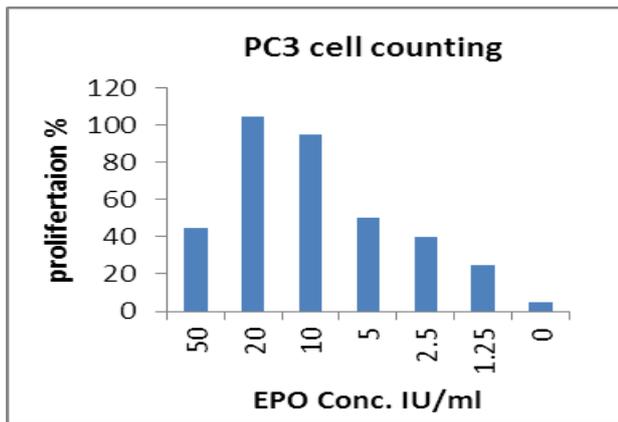
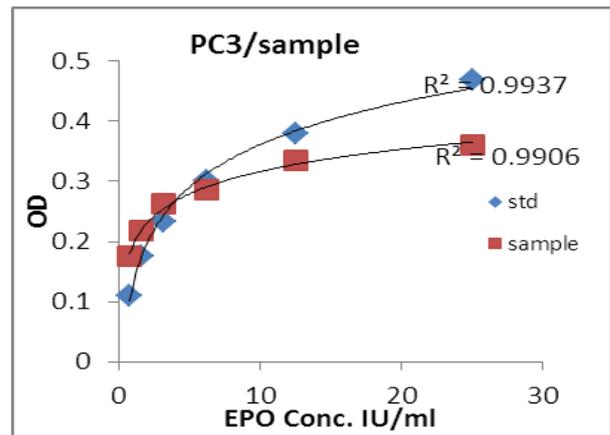


Figure 2: rhEPO induces proliferation % of Hela, HepG2, MCF-7 and PC3 cells treated with increasing concentrations of EPO as indicated. Cell proliferation was assessed at 72 hr using the MTT assay at 630 nm. Each point represents the average of three independent determinations.

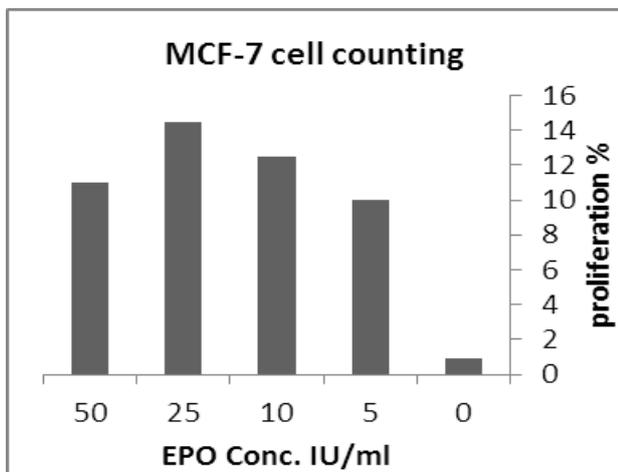


a)



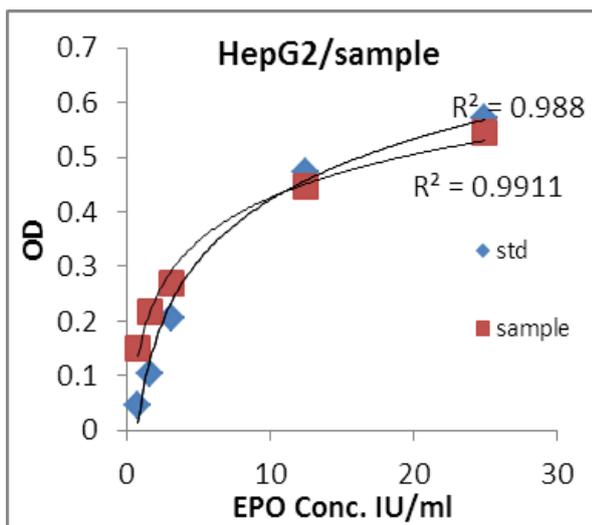
b)

Figure 4: rhEPO sample (Eprex 4000 IU/ml) bioassay using a) HepG2 cell line and b) PC3 cell line measured against EPO standard (120 IU/ml). Cell proliferation was assessed at 72 hr using the MTT assay at 630 nm. Each point represents the average of three independent determinations.

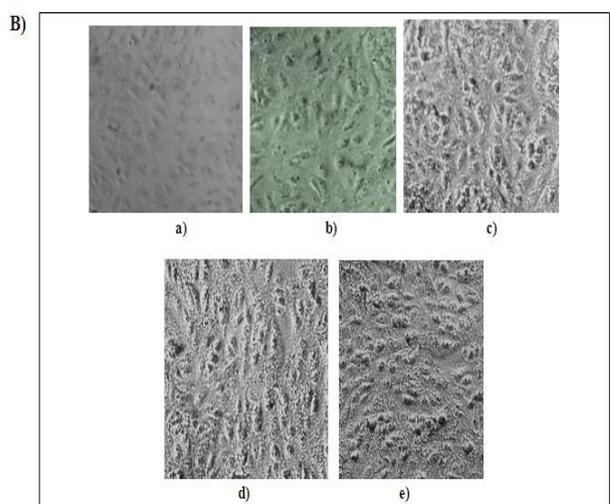
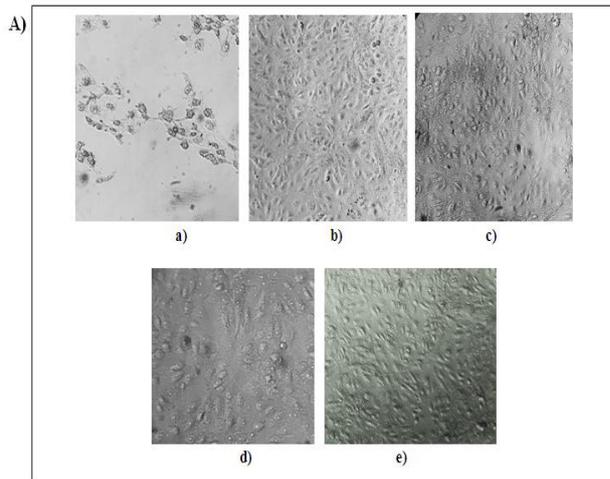


b)

Figure 3: rhEPO induces proliferation of a) PC3 cells and b) MCF-7 cells treated with rhEPO as indicated. The number of cells was counted by Neubauer chamber hemacytometer 72 hr post treatment.



a)



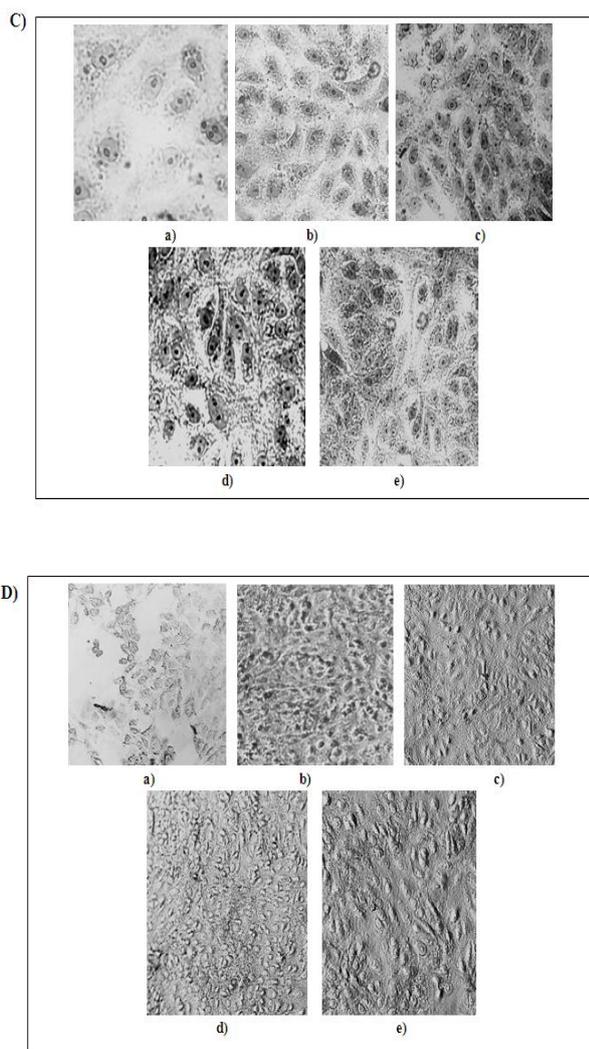


Figure 5: Microscopical examination shows rhEPO induced proliferation of A) HeLa, B) HepG2, C) MCF-7 and D) PC3 cells treated with rhEPO a) control non treated cells, b) 3.125 IU, c) 6.25 IU, d) 12.5 IU and e) 25 IU using SRB stain.

4. DISCUSSION

The pharmacological use of rHuEPO has been well determined with a clear impact on the life patients quality suffering from renal disease and those treated with chemotherapy.^[19] EPO is a glycoprotein, with approximately 40% of the molecular mass of the mature molecule made up of four carbohydrate chains.^[16] This protein structure should activate the EPO receptor which in cascade could be used for the evaluation of rhEPO bioassay *in vitro* as a routine quality control testing for batch release. This quality control testing overcome the drawbacks of other traditional methods and comply with US Pharmacopeia (USP) monograph <1032> Design and Development of Biological Assays) which states that Cell-based bioassays using clonal cell lines that respond to specific ligands or infectious agents can be used for lot-release assays of therapeutic proteins.^[17]

Identification of erythropoietin receptor was confirmed by immunohistochemical and immunochemical methods

for non-hematopoietic cells and different types of tumor cells^[20] Hence, functional erythropoietin receptor in tumor cells may lead to cancer cell proliferation^{[3], [21], [15], [6], [22], [7], [23]} This was confirmed in this study by investigating the proliferative effect of rhEPO on MCF-7, HELA, PC3 and HepG2 cell lines as shown in results. Binding of EPO to EpoR activates several signal pathways including JAK2-STAT5, Ras-MAPK and PI3K-ATK which transduce growth factors playing important role in cell survival, growth, and proliferation^{[24], [6], [25], [9], [8], [26], [27]}, Based on these mentioned findings, cell based bioassay could be used for lot-release assays of therapeutic EPO formulation.

Our study is strengthened by the using four separate tumor cell lines express functional EPO receptors on their surface and all show corresponding proliferation against increasing concentration of EPO up to 25 IU/ml, while excess amount of rhEPO affect the proliferation rate as demonstrated in the treatment of 50 IU/ml (fig.1,2 & 3). These results could assume that *in vitro* proliferation assay is rapid, sensitive and inexpensive to replace the current existing methodology for the EPO bioassay and overcome the drawbacks of the *in vivo* bioassay which restrict the further progress in this field.

5. CONCLUSION

The ability of rhEPO to stimulate the proliferation or viability of cells which express functional EPO receptors *in vitro* is planned to be used as a new methodology to assess the potency of rhEPO in pharmaceutical products from several manufacturers. The rhEPO test samples will be tested against primary international standard to ensure that, the bioactivity of EPO formulations are conformed to the specifications of the European Pharmacopoeia monograph of concentrated EPO solution <1316>, with potency values between 80 and 125% of the stated potency.

Determination of EPO bioactivity using cell culture assays is a simple, sensitive, rapid and well-characterized method to be applied for EPO formulations quality control testing aiming to ensure batch-to batch consistency among the different manufacturers in order to guarantee high quality and therapeutic efficacy.

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