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Enhancement of Tissue Type Plasminogen Activator (tPA) Production from Recombinant CHO Cells by Low Electromagnetic Fields

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Abstract Low Electromagnetic Field (EMF) intensity in the range of 1 μT to 10 μT (Tesla) was found to enhance the growth of CHO cells and the production of tPA in batch and perfusion cultivations. At 1 μT intensity, 1.3×10^7 viable cells/ml of maximum cell density and 80 mg/l of maximum tPA production were obtained in batch cultivation, compared to 2.8×10^6 viable cells/ml and 59 mg tPA/l in unexposed case (control). A similar trend was observed in the perfusion process, where it was possible to obtain 1.2×10^7 viable cells/ml of maximum cell density and 81 mg tPA/l of maximum tPA production by more than 80 days of cultivation. However, there was not much difference between 1 μT and 10 μT in perfusion cultivation, possibly due to better environmental growth conditions being maintained by continuous feeding of fresh medium into the reactor. On the contrary, both cell growth and tPA production were severely inhibited at higher than 1 mT intensity, showing no growth at 10 mT exposure. Specific growth rate was linearly correlated to specific tPA production rate at 1 μT EMF intensity, which represents a partially growth-related relationship. It was also found that a large amount of Ca^{2+} was released at low EMF intensity, even though the cell growth was not much affected. Low EMF intensity significantly improved both cell growth and tPA production, and tPA production seemed to be more affected than the cell growth, possibly due to the changes of cell membrane characteristics. It can be concluded that the elaboration of EMF intensity less than 10 μT could improve cell growth and tPA production, but mainly tPA secretion through batch or perfusion process in a bioreactor.

Key words: Tissue type plasminogen activator, recombinant CHO cells, electromagnetic fields

There have been many reports on the effects of electromagnetic fields (EMFs) on inducing human diseases [4, 15, 24]. They are mostly epidemiological and clinical studies related to cancers, and some with cellular studies [7, 13, 28]. The EMF intensity used in the experiments are in the range of 10 μT (Tesla, 1 μT=0.01 Gauss) to 100 mT at 60 Hz, which is significantly higher than those from the naturally developing fields of 1–7 μT [1, 2, 27]. Therefore, high EMFs certainly appear to cause or be related to inducing diseases. However, convincing evidence of the EMF-related effects on the pathways of regulating cell proliferation and cell growth is needed, even though several results about the effects on cell growth have been reported [4, 19, 16, 30]. Almost all these experiments failed to cover all aspects of EMF-induced biological responses. There is ongoing controversy on the effect of short or long exposure to very low EMFs on human pathology, because there has been a lack of plausible biological mechanisms to explain how such exposure can affect the biological systems, negatively or positively [14, 20, 26].

There have been great advances in the development of culture systems for growing large amounts of mammalian cells for eventually producing economical amounts of proteins [5, 9, 18, 21, 23]. The limit may have been reached for developing new culture systems to dramatically improve the productivity, when only physical culture systems are considered. Therefore, the importance of interplay between cell-to-cell and cell-to-reactor is getting more attention to overcome the bottlenecks of enhancing protein productivity [10, 18, 31]. However, employing EMFs in producing and/or secreting proteins of interest in *in vitro* cultures of mammalian cells has never been tried. Therefore, it is necessary to conduct *in vitro* laboratory experiments in order to find out how low EMFs affect the cell culture system by influencing signal transduction, cell membrane, and cell proliferation, etc. [6, 8, 12, 17]. It may provide a way of utilizing EMFs in improving cell culture performance, yielding no negative effects on cell culture systems. In this work, recombinant cells were exposed to very low EMFs in a culture system, which could affect protein productivity,

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because of changes of the cell growth at both the cellular and molecular levels. Additionally, it will provide a way of producing large amounts of biopharmaceuticals *in vitro* from mammalian cells in an EMF-controlled bioreactor.

MATERIALS AND METHODS

Cell and Culture Condition

Recombinant Chinese Hamster Ovary (CHO) cells producing tPA (ATCC, CRL 9606, U.S.A.) were grown in DMEM/F12 (Sigma, St. Louis, U.S.A.) basal medium containing 40 nM methotrexate (Sigma, U.S.A.), 20 mM glucose, 365 mg/l of L-glutamine (GIBCO, New Jersey, U.S.A.), and enriched with 10% FBS (GIBCO, New Jersey, U.S.A.) in a 37°C CO_2 incubator supplying 5% CO_2 in air. The cells were maintained in a 75 cm^2 T-flask, and 1×10^5 viable cells/ml were inoculated into a bioreactor (working volume, 2 l, KoBiotech., Inchon, Korea), which contained 8 g/l of microcarriers (Cytodex III, Sigma, U.S.A.) in 500 ml of preheated medium. After inoculation, the cells were cultured for 4 h to attach cells to the microcarriers, then the rest of the prewarmed medium (ca. 1.5 l) was added into the reactor and agitated at 50 rpm. After the cell growth was reached to exponential phase, 5 g/l of beads was additionally added into the reactor through a medium feeding line after stopping the agitation. Then, the reactor was slowly agitated for 1 h. Air containing 5% CO_2 was diffused into the medium by a silicone tubing located between the EMF generator and an impeller inside the reactor (Fig. 1). pH and D.O. was automatically adjusted to 7.0 and 25% air saturation, respectively, by a microprocessor attached to the reactor. For perfusion cultivation, the level of medium was controlled by a level controller when fresh medium was perfused by a peristaltic pump. The conditioned medium was also removed by a peristaltic pump through a 50 μm pore size decanting filter cartridge to recycle the cells into the reactor. Perfusion rate was changed depending on cell growth by measuring cell density every day. Each run had been operated for about 80-90 days for perfusion cultivation until the cell growth reached to stationary phase. Then, the system was shut down and other runs were prepared.

A silicone-coated waterproof EMF generator was located inside the reactor, which consisted of 2,500 turns of copper magnet wire in a near Helmholtz configuration, as shown in Fig. 1. The power was supplied by an AC voltage supplier (Kangdong Ind., Seoul, Korea) varying the current at 55-60 Hz frequency. The magnetic flux density was measured by a magnetometor (9200, F.W. Bell Inc., Chicago, U.S.A.) and the geomagnetic field without power to the coil was negligible. The EMF intensity was changed by controlling the output of the audio signal and sinusodial generator (DSG2, SETOL Co., Campbell, U.S.A.). Once all of the medium had been fed into the bioreactor, the culture system was agitated and also continuously exposed to various EMF intensities (1 μT, 10 μT, 1 mT, and 10 mT, respectively).

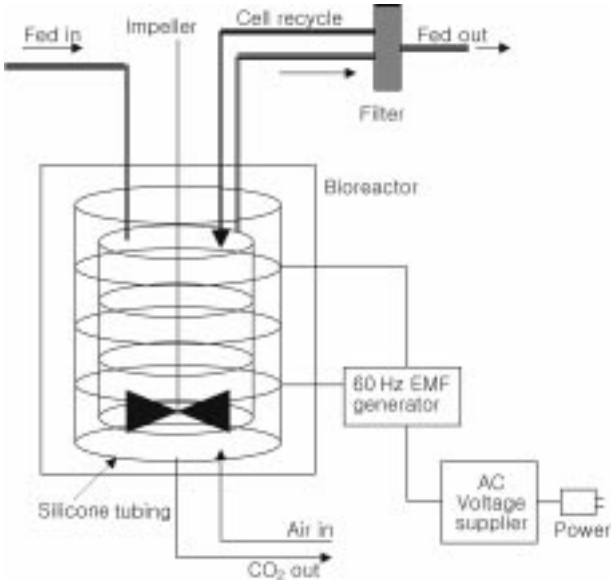

Fig. 1. A diagram of a bioreactor equipped with an EMF-generating apparatus.
The apparatus was located inside of the bioreactor. Aeration was made by silicone tubings located between an impeller and an EMF generator.

Measurement of Cell Concentration and Biochemical Analysis

Cell density was measured using a hemocytometer after detaching the cells from the beads by treating them with 5 ml of trypsin solution (200 unit/ml, 1 mM EDTA, Sigma, U.S.A.) for 20 min. The cell viability was estimated everyday by the trypan blue dye exclusion method [11]. tPA concentration in the conditioned medium was measured by using a chromogenic enzymatic method as follows [32]: The medium was incubated with plasminogen (10 unit, Sigma) and chromogenic peptide, D-Val-Leu-Lys-*p*-nitroanilide (S-225, Sigma), for one hour at 37°C. The absorbance of generated para-nitroanilide was measured by an ELISA reader (Bio-RAD, Georgia, U.S.A.) at 405 nm. Glucose concentration in the medium was estimated by a glucose kit (Kyokuto Pharmaceutical Co., Kyoto, Japan) at 560 nm using a spectrophotometer.

Cytosol Ca^{2+} concentration released from the cells was measured as follows: The collected cells were suspended with 2 ml of Na^+ solution containing 135 mM NaCl, 5 mM KCl, 1 mM MgCl, 10 mM glucose, and 20 mM HEPES. The fluorescent intensity (F) of the suspension was measured by a fluorescent spectrophotometer (Perkin Elmer, New York, U.S.A.) at 331/398 nm (excitation/emission). The Ca^{2+} ion concentration in the cytosol, $[Ca^{2+}]$, was calculated as follows:

$$[Ca^{2+}]=K_d(F-F_{min})/(F_{max}-F) \quad (1)$$

where K_d is the Ca^{2+} dissociation constant in Indo-1 solution (250 nM), F_{max} is the fluorescent intensity of the suspension measured before being exposed to EMF, and F_{min} is the fluorescent intensity of Indo-1 solution without Ca^{+2} calculated by the following equation (2).

$$F_{min}=(1/12)(F_{max}-F_{auto})+F_{auto} \quad (2)$$

F_{auto} was a measure of the Indo-1 solution, with only 2 mM $MnCl_2$ added, at 331/398 nm [29].

The specific growth rate, μ (1/day), and tPA production rate, Q_p (mg tPA/cell/day), were estimated by the following equations:

$$\mu=(1/X)(dX/dt) \quad (3)$$

$$Q_p=(1/X)(dP/dt) \quad (4)$$

where X is cell density (viable cell/ml), t is cultivation time (day), and P is tPA concentration (mg/l). The relation between cell growth and tPA production was also correlated by the following equation based on the partial growth-related tPA production process from recombinant CHO cells [22, 25]:

$$Q_p=\alpha\mu+\beta \quad (5)$$

where α is tPA production rate per cell (mg tPA/cell) and β is tPA production rate (mg tPA/cell/day) [3].

All the experiments were carried out at least three times. The data points in the figures are the mean averages of the experiments and the bars are mean standard deviations calculated by the Statistical Analysis System (SAS, N.C., U.S.A.).

RESULTS AND DISCUSSION

Effect of EMFs on Cell Growth and tPA Production in Batch Cultivation

Figure 2 shows the effect on cell growth and tPA production by EMF intensities for batch cultivation. It is evident that both the cell growth and tPA production were greatly improved by being exposed to 1 μT of low intensity EMF. Slight enhancement of cell growth and tPA production was also observed at 10 μT of EMF intensity, compared to the control (unexposed case). It is interesting to note that tPA production remained steady at latter periods of cultivation, even though the cell growth started to decrease at 1 μT and 10 μT EMF intensities, while tPA production decreased in the control and at high EMF intensities. Cell growth seemed to be more sensitively affected than tPA production, when exposed to low EMF intensity, by showing that the cell growth was greatly increased up to 1.3×10^7 viable cells/ml vs 1.28×10^6 viable cells/ml for the control. Maximum tPA production was 80 mg/l vs 59 mg/l at 1 μT of low EMF intensity and for the control, respectively. This shows that the enhancement of tPA production at low EMF intensities

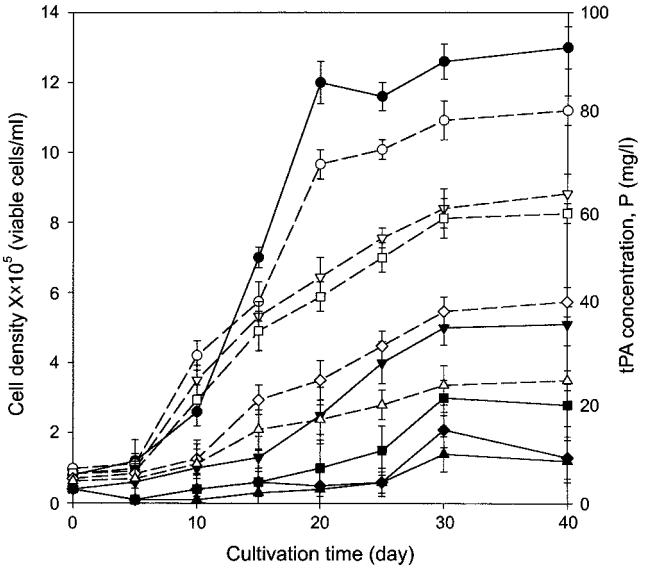

Fig. 2. The growth of recombinant CHO cells and the production of tPA under various EMF intensities, under batch cultivation.
Cell density, X: Control (■), EMF 1 μT (●), EMF 10 μT (▼), EMF 1 mT (◆), EMF 10 mT (▲); tPA concentration, P: Control (□), EMF 1 μT (○), EMF 10 μT (▽), EMF 1 mT (◇), EMF 10 mT (△).

was partially due to the improvement of cell growth. At high EMF intensities, the cell growth was much decreased, compared to that for the control. However, residual concentration of tPA in the medium seemed to be increased, possibly because of increased secretion of tPA from the cells due to malfunction of the membrane at high EMFs. tPA production was also much affected at 1 mT or 10 mT of high EMF intensities, showing a sudden drop in production at a latter period of cultivation when the cell growth decreased. tPA production was not observed at the highest EMF intensity of 10 mT due to severe inhibition of cell growth.

Figure 3 illustrates the pattern of glucose consumption in the medium during batch cultivation of CHO cells when exposed to various EMF intensities. Relatively high glucose consumption was observed at low EMF intensity of 1 μT, which correlated well with the cell growth in Fig. 2. Very small amounts of glucose were consumed at high EMF intensity, compared to the control. For the unexposed case, residual glucose concentration at a latter period of cultivation was similar to that at low EMF intensity. As shown in Fig. 2, a significantly fast consumption rate was observed at exponential growth phase at low EMF intensity, compared to that for the control, and the tPA production seemed to be well correlated to the glucose consumption.

Effect of EMFs on the Cell Growth and tPA Production in Perfusion Cultivation

Figure 4 shows the result of cultivating CHO cells under perfusion condition for about 80 days. In perfusion

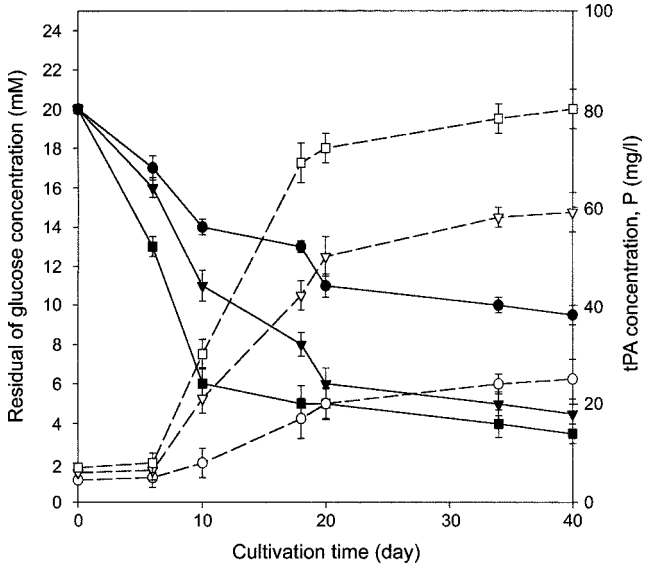

Fig. 3. Kinetics of glucose consumption and tPA production from recombinant CHO cells being exposed to EMF intensity under batch cultivation.
Residual glucose conc, S: Control (▼), EMF 10 mT (●), EMF 1 μT (■); tPA concentration, P: Control (▽), EMF 10 mT (○), EMF 1 μT (□).

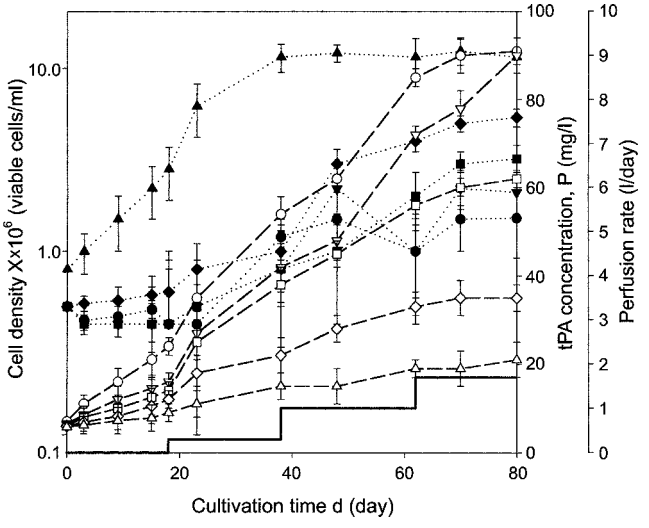

Fig. 4. Recombinant CHO cell growth and tPA production under various EMF intensities in perfusion cultivation.
Cell density, X: Control (■), EMF 1 μT (▲), EMF 10 μT (◆), EMF 1 mT (▼), EMF 10 mT (●); tPA concentration, P: Control (□), EMF 1 μT (○), EMF 10 μT (▽), EMF 1 mT (◇), EMF 10 mT (△).

cultivation, the cell growth was much better at 10 μT intensity than that at same intensity in batch cultivation, maintaining ca. 5.8×10^6 viable cells/ml of maximum cell density in perfusion cultivation and 5.1×10^6 viable cells/ml in batch cultivation, as shown in Fig. 2. tPA production was also greatly enhanced at 10 μT intensity, yielding ca. 81 mg/l of maximum production in perfusion cultivation vs 63 mg/l in batch cultivation. It could be that the continuous feeding of fresh medium maintained better environmental conditions for growth and partially decreased the negative effects of being exposed to EMFs. This also resulted in prolonging the overall cultivation time in the perfusion process by controlling the perfusion rate, which resulted in increased specific growth rate. However, at 1 μT of low EMF intensity, both cell growth and tPA production were not significantly improved, compared to that in batch cultivation at the same EMF intensity. For the case of the control, cell growth increased in the perfusion cultivation compared to that in batch cultivation, as shown by 4.1×10^6 viable cell/ml vs 2.8×10^6 viable cells/ml, respectively, whereas tPA production was not much improved, as shown by 62 mg/l vs 59 mg/l, respectively. This implies that perfusion cultivation can definitely have a masking effect on EMF exposure for enhancing cell growth, but does not directly affect tPA production at low EMF intensity. However, it was of interest to observe that the perfusion process positively affected both cell growth and tPA production at relatively high EMF intensities. In particular, the cell growth at 10 mT did not decrease during the cultivation, while the cells suddenly stopped growing in batch cultivation. tPA production did not decrease at either 1 mT or 10 mT intensities. This may also indicate that cell growth and tPA production could be improved and/or protected in perfusion cultivation by being exposed to high EMF intensities.

Continuous production of tPA was also observed at a later period of cultivation, which did not occur in batch cultivation. Table 1 shows estimating growth parameters obtained from batch and perfusion cultivations at high and

Table 1. Result of estimating growth parameters by being exposed to two different EMF intensities.

	Control			EMT 1 μT			EMF 10 mT		
	Maximum cell density, X (viable cells/ml)	Specific growth rate, μ_{max} (1/day)	Maximum tPA production, P (mg/l)	Maximum cell density, X (viable cells/ml)	Specific growth rate, μ_{max} (1/day)	Maximum tPA production, P (mg/l)	Maximum cell density, X (viable cells/ml)	Specific growth rate, μ_{max} (1/day)	Maximum tPA production, P (mg/l)
Batch	2.8×10^6	0.17	59	1.3×10^7	0.45	80	1.1×10^6	0.06	24
Perfusion	3.1×10^6	0.19	62	1.2×10^7	0.59	81	1.2×10^6	0.06	22

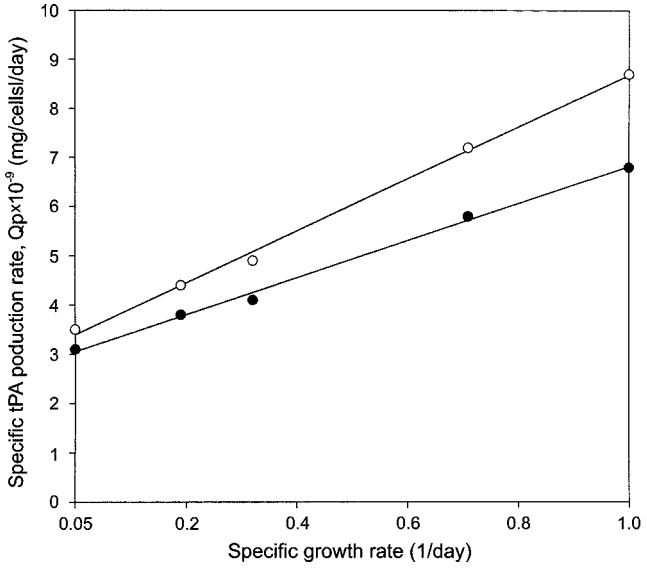

Fig. 5. Result of correlating specific tPA production with specific growth rate from the data in perfusion cultivation.
One μT EMF intensity (○), Control (●). The line is the result of linear regression.

low EMF intensities. It was evident that both maximum cell density and tPA production were improved by being exposed to low EMF intensity, compared to those from the control. In general, tPA production seemed to be much more enhanced than cell growth at low EMF intensity. The perfusion process also proved to be a better culture system for increasing tPA production at low EMF intensity than the batch process, based on the enhancement of cell growth and tPA production. At very high EMF intensity, the maximum cell density was the same as the initial cell density, demonstrating that the cell could not grow at all under these conditions and resulted in no tPA production.

Figure 5 illustrates the relationship between specific growth rate and tPA production rate in perfusion cultivation estimated by employing Eq. (3). Both experimental data seemed to be well correlated to Eq. (3) by yielding 0.93 and 0.89 of correlation factor for the control and at 1 μT EMF intensity, respectively. It is highly likely that tPA production related strongly to cell growth. This also indicates that the enhancement of tPA production was mostly due to the increase of cell growth at 1 μT EMF intensity, compared to the control. This hypothesis was also supported by the results that tPA production was greatly reduced by the decrease of cell growth at high EMF intensities. The tPA production rate per cell and the specific tPA production rate were estimated from the linear portion in Fig. 5 to be 4.89×10^{-9} mg tPA/cell and 3.51×10^{-9} mg tPA/cell/day, respectively, at 1 μT EMF intensity and 3.68×10^{-9} mg tPA/cell and 3.13×10^{-9} mg tPA/cell/day for the control. This implies that low EMFs could trigger the enhanced release of tPA production from the cells by increasing the tPA production ten times more than the control in a single cell. This would also result in increasing the specific tPA production rate in perfusion cultivation.

The Relationship Between Cytosolic Ca^{2+} Ion and tPA Production

Figure 6 shows the mechanism of how tPA production could be increased at low EMF intensity, by measuring cytosolic free calcium ion, since Ca^{2+} in the cytosol was released from the cell due to environmental changes [5, 21]. The degree of released Ca^{2+} ion could affect the release and/or secretion of tPA from the cell, which eventually increased the production of tPA in the reactor as shown in Fig. 6. It was proven that low EMFs could increase the growth of recombinant cells and possibly resulted in the increase of tPA production. In Fig. 6, the degree of released Ca^{2+} ion was greatly increased during cultivation by being exposed to 1 μT intensity, and the increase of tPA production also correlated to the release of Ca^{2+} ion. This supports the idea that low EMFs could have an affect on increasing both cell growth and secretion of tPA out of the cell. Low EMFs seemed to be more effective at releasing tPA from the cells rather than increasing cell growth, according to the data in Figs. 4 and 6. However, this result did not cover all aspects of EMFs effect on increasing all other proteins in the cells. Further research on the production of proteins at the cellular and molecular levels under the influence of low EMFs is needed.

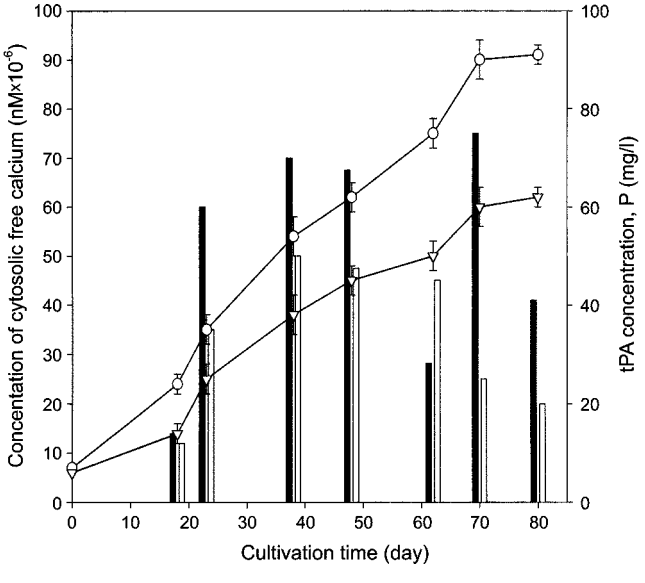

Fig. 6. The concentration of cytosolic free calcium and tPA production from recombinant CHO cells at 1 μT EMF intensity or exposure according to cultivation time.
Cytosolic Ca^{2+} ion : Control (■, bar), 1 μT (□, bar); tPA concentration, P: Control (○, line), 1 μT (▽, line).

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