Research Article/REVIEW ARTICLE/Case Report/General Article

TAMOXIFEN EFFECTS ON GENE EXPRESSION OF NEUROPROTECTIVE FACTORS

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Please Include Suheadings shaded with Yellow colour in Your Article. Article such as Review article, Mathematical derivation don't need to write MATERIALS AND METHODS

ABSTRACT

Astrocytes are the most glial cells in central nervous system those secrete protective factors. Tamoxifen is a non steroid drug and an antagonist of the estrogen receptor and was used in breast cancer treatment. In the present study the possible effects of tamoxifen on the morphology of astrocytes were investigated *in vitro*. After separating the cortex and tissue trituration of two rat embryo (wistar) were cultured in Dulbecco's Modified Eagle. Tamoxifen (8, 12 and 16) µmol/L was added to the cultured astrocytes for 72 hours. The cells were purified by trypsinization, determined and evaluated by immunocytochemistry technique. Astrocytes' invert electron microscope and gene expression of neuropr was determined. The Expression of the above-mentioned facto nique and qualified the rate of them by PCR Real-time. The m rescence and data analyzed by statistical SPSS (18) soft ware. re much higher amounts of TGFβ1 and TGFβ2 in comparison ant difference was not observed in the β actin expression (P< . expression of neuroprotective activity of astrocytes. Also, it can

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Keywords: *Astrocytes,*

INTRODUCTION

The glial cells (neuroglia) su the neuaral cells and are useful for nutrition, activity and they have a role in the regulation of re neurons after injury in the central nervous system (Allen and Barres, 2005). The glial cell exc Schwann divided to three types as oligodendrocyte, microglial and macroglial. Astrocytes are characteristic star-shaped the most abundant type of macroglial cell in the cortex[1] **(Seth and Koul, 2008) [PLEASE REPLACE ALL NUMERIC VALUES/NUMBERS (IF WRITTEN) WITH AUTHORS SURNAME AND YEAR].** They have numerous projections for anchor neurons to their blood supply (Koehler *et al.*, 2009).

They also have aqua porin (AQP) that makes them permeable to ions and various molecules as nucleotides, glucoside, peptide, cAMP, calcium, inositol and amino acids. They perform biochemical support of endothelial cells that form the blood–brain barrier, maintenance of extracellular ion balance, and a role in the repair and scarring process of the brain and spinal cord injuries (Volterra and Steinhäuser, 2004; Allen and Barres, 2005).

Recent studies have shown that astrocytes play an important function in the regulation of neural stem cells. They are able to activate the stem cells to transform into other neurons (Horner and Palmer, 2003). In brain injuries, these cells become active and will change morphologically **(Aschner *et al.*, 2002).**

One of the most important functions of astrocytes is to secrete neuroprotective factors which are effective in neural survivals and regenerations of neurons. These factors are TGFβ (Transforming growth factor), BDNF (Brain-derived neurotrophic factor), Interleukin-10 and NGF or nerve growth factor (Dwivedi, 2009; Yan *et al.*, 2009). Tamoxifen is an antagonist of the estrogen receptor in breast tissue via its active metabolite, hydroxytamoxifen. In other tissues such as the endometrium, it behaves as an agonist, and thus may be characterized as a mixed agonist/antagonist (Krishnan *et al.*, 2003). So it is a useful drug

for breast cancer treatment and ovulation. As it has antagonistic effects on hypothalamus and pituitary gland, it can pass the blood-brain barrier (Patterson *et al.*, 1981). TGFβ which is isoform of TGF (Transforming growth factor) is in brain. Its isoform have two kinds of receptors (TβR-I TβR-II). TGF isoforms are active microglia. These factors are important in treatment of neural cells (Ronaldson *et al.*, 2009). GFAP increase in neural damages as Alexander disease (Brenne *et al.*, 2001). Other investigates have showed that BDNF and vascular endothelial growth factor (VEGF) contribute to neurovascular after stroke, and these responses involve both recovering endothelium and reactive astrocytes (Xiu *et al.*, 2009). The effect of tamoxifen was studied in different organs previously (Ruffy, 2006; Wakade *et al.*, 2008). In this study, the effects of it on expression of neuroprotective factors (BDNF, $TGF\beta_1$ ،$TGF\beta_{2)}$ which were secrated by astrocyte, was determined by PCR.

MATERIALS AND METHODS THIS SUBHEADING IS NOT REQUIRED IN ARTICLE WITH MATHEMATICAL DERIVATIONS AND REVIEW ARTICLE

To do the research, the first step was culture astrocytes. For this purpose, all the devices and the laminar flow hood were sterilized in 160°_{c}, the skin of two rat emberio (wistar) was disinfected and its head was cut. Then the skull was dissected from neck to frontal. After separating the cortex without any membrane, it was cleaned with Hank's buffered saline solution (HBSS), then was cultured in DMEM medium (Dulbecco's Modified Eagle) which include FBs 10%. Obtain cell were separated by trituration and transferred to DMEM. These cell after polilifratin and transferred. These cells were purified with trypsinization after one week and transferred to other flask. The isolated astrocytes were stained with triphan blue and counted with Neubauer slide. In the second step, the expression of GFAP (Glial fibrillary acidic protein) which is special marker for astrosyte by immunocytochemistry was investigated. To study this step, the cells cultured on the cover slips and was done as follow: The astrosytes were washed with PBS, fixed in paraformaldehyde 4% (15 minutes), cleaning with PBST (PBS+Tween), incubation in PBST 10% (was prepared from serum goat) in the room temperature for 45 minutes, Incubation these cells with antibody (GFAP) in 37°_{c} for 60 minutes menthes, washing with PBST and studied by fleurscene microscope. In third step of experiment, the cultivated astrocytes were treated with (8, 12, and 16) µmol/L of tamoxifen for 72 hours. After treatment period, the cells separated by tripsin and RNA were extracted.
CDNA synthesis as follow:

Table 1: Program of cDNA synthesis

dNTP	dH2O	Buffer (10x)	$MgCl_2$	Taq plymerase	Primer – Reverse(R)	Primer-Forward(F)	cDNA
5 µl	9 µl	3 µl	2 µl	5 µl	1 µl	1 µl	5 µl

70 ºC: 10', 42ºC: h2 ،25ºC: 10' و 70ºC: 5'

RT-PCR was done in later step. B-actin primer was known as control.

Table 2: Program of cDNA synthesis by **RT-PCR**

dNTP	dH2O	Buffer (10x)	$MgCl_2$	Taq polymerase	Primer – Reverse(R)	Primer-Forward(F)	cDNA
2 µl	9 µl	3 µl	1 µl	5 µl	1 µl	1 µl	5 µl

95ºC: 5', 60 ºC: 17', 72ºC: 1', 72 ºC: 1'

After doing PCR, the primer sequence was determined and genes expressions were confirmed by RT-PCR. Data were obtained (Pfaffl *et al.*, 2003) and was calculated ratio by below formula:

$$\text{ratio} = \frac{(E_{\text{target}})^{\Delta Ct\ \text{target (control-treated)}}}{(E_{\text{ref}})^{\Delta Ct\ \text{ref (control-treated)}}}$$

Threshold Cycle (CT), Efficiency (E)
The end f experiment, statistical soft ware (SPSS) and test ANOVA (P< .001) were used and data analyzed.

RESULTS AND DISCUSSION

Table 1: The gene expression of TGFβ$_1$ [PLEASE STRICTLY FOLLOW THE TABLE FORMAT]

Tamoxifen	Ratio						Mean±SE	SE
8 µmol/L	3.16	4.92	4.22	2.19	5.98	1.75	3.7033	.66662
12 µmol/L	5.58	6.27	8.54	7.01	6.81	5.64	6.6417	.44856
16 µmol/L	7.26	8.13	9.05	8.6	7.9	8.03	8.1617	.25041

Table 2: The gene expression of TGFβ$_2$ in tomxiphen treated cells

Tamoxifen	Ratio			Mean±SE	SE
8 µmol/L	3.46	4.93	4.94	4.4433	.49168
µmol/L 12	8.42	7.11	5.82	7.1167	.75056
16 µmol/L	8.95	9.38	7.87	8.7333	.44916

Table 3: The gene expression of BDNF in tomxiphen treated cells

Tamoxifen	Ratio			Mean±SE	SE
8 µmol/L	2.57	2.97	3.19	2.9100	.18148
µmol/L 12	4.91	4.25	3.96	4.3733	.28109
16 µmol/L	7.19	7.70	8.82	7.4450	.25500

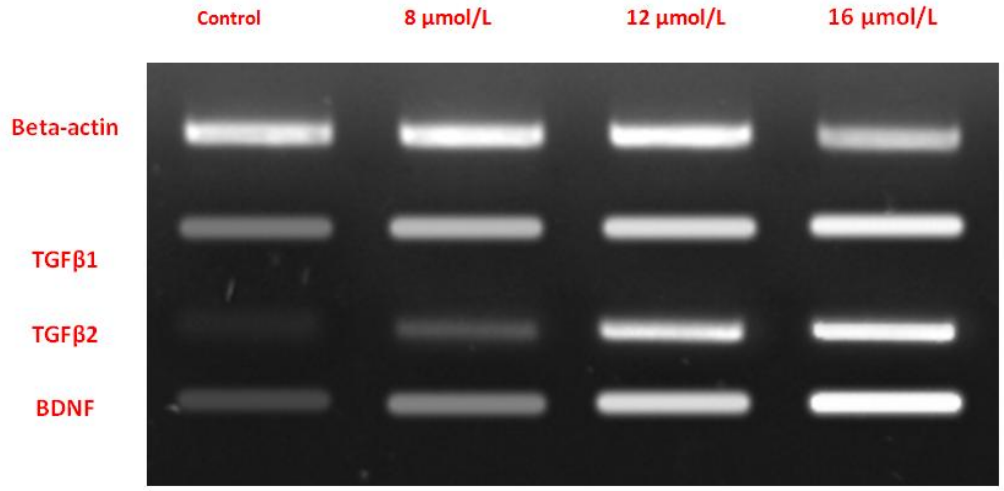

Figure 1: The effect of tamoxifen on expression of neuroprtective factors in Rat embryo
FIGURE MUST BE NUMBERED WITH TITLE

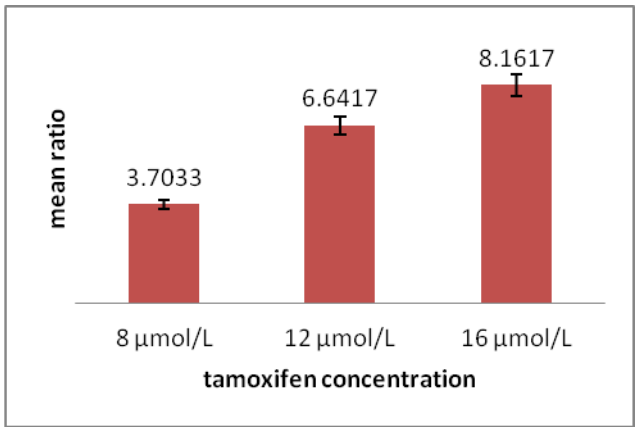

Figure 2: The expression of neuroprotective factor ($TGF\beta_1$)

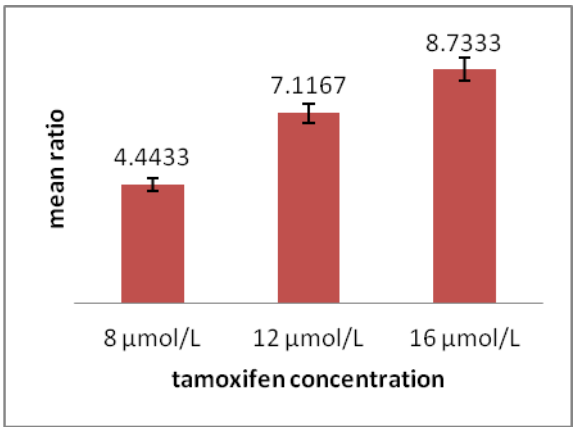

Figure 3: The expression of neuroprotective factor($TGF\beta_2$)

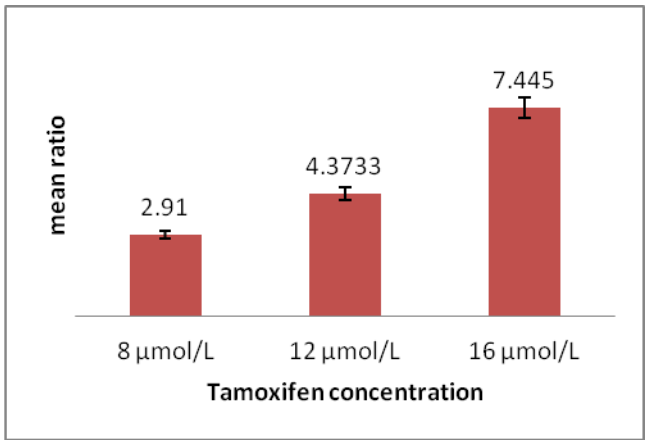

Figure 4: The expression of Brain-derived neurotrophic factor (BDNF)

Results

This study showed that tamoxifen increased the expression of BDNF, $TGF\beta_1$, $TGF\beta_2$ which were secreted by astrocytes and this effect was dose- dependent. Also was shown that this drug didn't affect the expression of β actin. The obtained results of real time PCR were demonstrated in tables (1-3) and Figures (1-4).

Discussion

If injuries happen in the central nervous system, physiologic mechanisms will be activated and one can refer to the glial cells (Yan *et al.,* 2009). Astrocytes are the most effective to control of neural cell and its survival by secretion of the factors such as TGFβ1, TGFβ2, GDNF, and NT3 (Seth and Koul, 2008; Siegel *et al.*, 2000).

The researchers isolated astrocyte from either enzymatically or mechanical methods. Mechanical method depending on size of cell and based on different adhesion properties, divided to two ways (Lim and Miller, 1989). In this study, astrocytes isolated by mechanical method, because these cells have high adhesion property and prepared high purely. Also in this method the membrane of cells don't damage.

Although astrocytes were isolated from different part of CNS (Barres, 2008), but numerous of them were located within white matter as cortex. On the other hand, the neuroprotective factors which were important in proliferation, differentiation and protection of neurons are secreted by astrocytes (Broer *et al.*, 2001; Ronaldson *et al.*, 2009).

According to the obtained results in this study, tamoxifine increased expression of these protective factors and effect of it, depended to dose of drug consume (Mikulec *et al.*, 2000). The previous investigates showed that the some compound such as 17β-Estradiol (E2) and selective estrogen receptor modulators (SERMs), such as tamoxifen, mediate numerous effects in the brain, including neurosecretion, neuroprotection, and the induction of synaptic plasticity (Naoko and Shinichi, 2003; Ronaldson *et al.*, 2009).

Previous reports showed that tamoxifen is neuroprotective against apoptotic cell death via ER-dependent mechanism in Rat (Zhang *et al.*, 2007). The astrocytes have receptors with kinase property those active signaingl pathways (Wang *et al.*, 2006). They activate PI3K/Akt pathway, PI_3 kinase, phospholipase C and proto-oncogene that become an oncogene due to mutations or increased expression (Hanada *et al.*, 2004; Dhandapani *et al.*, 2005). The WNT pathway is the most of them and it encode a large family of secreted protein growth factors such as TGF-Betas (Transforming Growth Factor-Betas), FGFs (Fibroblast Growth Factors), Hedgehog and Notch proteins that have been identified in animals from Hydra to Human. Wnt signaling pathway was actived via inhibiting of enzyme GSK-3β (Glycogen synthase kinase 3 beta) that it is a key molecule (Koh *et al.*, 2004).

Whereas seems in present research, tamoxifen effects on cortical astrocytes via PI3K/Akt pathway and inhibiting GSK-3β enzyme that due to increasing of neural protective cells ($TGF\beta_1$, $TGF\beta_2$). Since it is the most important endocellular signaling pathway in cell division, cell migration, cell survival, determination and its disorder cause neurodegenerative diseases (Lu *et al.*, 2005; Liu *et al.*, 2003). The inhibitor induced IGF expression and activation Wnt pathway that increase neuron survival (Koh *et al.*, 2004). The GSK3β is important in growth factor secretion and these factors are effective on signaling pathway. Moreover TGFβ and BDNF receptors are in astrocytes. So we can say that tamoxifen increase the above factors via inhibit GSK3β.

Conclusion

Cortical astrocytes could provide a mechanism of neuroprotection, and that tamoxiphen stimulation of TGF-β expression. Tamoxifen increase expression of TGFβ and BDNF that probably act via direct impact or increased activity of Wnt signaling pathway by inhibiting the GSK3β. Therefore, this pathway can be activated by using agonists as tmoxifen and increased neural expression of protective factors. Since these factors can play a tremendous impact on neural survival and recovery of damaged CNS.

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