UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
INSTITUTO DE BIOCIÊNCIAS
CURSO DE CIÊNCIAS BIOLÓGICAS

NEUROPROTECTIVE EFFECT OF 17β-ESTRADIOL IN ORGANOTYPIC SLICE CULTURES OF RAT HIPPOCAMPUS EXPOSED TO OXYGEN AND GLUCOSE DEPRIVATION.

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E a todos que eu possa ter esquecido...

Lista de Abreviaturas:

AE – Acute Estrogen treatment

Akt/PKB – Akt/Protein Kinase B

C - control

CA1 - "Cornus Ammonis 1"

CE – Chronic Estrogen treatment

DMSO - Dimethylsulfoxide

ECL – Enhanced Chemio Luminescence

ED – Estrogen with DMSO

ER – Estrogen Receptor

GSK-3β - Glycogen synthase kinase - 3β

HBSS – Hank's balanced salt solution

HEPES – N-2-hydroxyethyl piperazine N-2-ethanesulfonic acid

MAPK – Mitogen-activated protein Kinase

MEM – Minimum Essential Media

MTTBS – Milk-T-TBS

NMDA – N-methyl-D-aspartate

OGD – Oxygen Glucose Deprivation

PAGE - Poliacrylamida Gel Electrophoresis

p-GSK-3β - Phospho- Glycogen synthase kinase - 3β

PI – Propidium Iodide

PI-3K – Phosphatidylinositol – 3Kinase

TBS – Tris Buffered Saline

TTBS – Tween – TBS

SDS – Sodium Dodecil Sulfate

Resumo

O cérebro é altamente dependente de um fluxo sanguíneo contínuo para suplemento de glicose e oxigênio. A isquemia cerebral resulta na degeneração celular e na perda da sua funcionalidade. Culturas organotípicas representam um modelo *in vitro* que permite estudar eventos e mecanismos envolvidos com o dano cerebral isquêmico. Neste trabalho nós investigamos o efeito neuroprotetor do 17β-estradiol em um modelo de isquemia *in vitro*. Para mimetizar um insulto isquêmico, fatias de cultura organotípica foram expostas à privação de oxigênio e glicose (OGD – Oxygen and Glycose Deprivation), usando uma câmara anaeróbica desenvolvida em nosso laboratório. A morte celular foi quantificada pela medida da captação do corante Iodeto de Propídio. Foi observada uma diminuição na incorporação de iodeto de propídeo nas fatias submetidas a OGD que receberam tratamento agudo e crônico com 17β-estradiol (10nM). Para examinar um possível mecanismo pelo qual 17β-estradiol previne a morte celular nós avaliamos o imunoconteúdo de pGSK-3β por *Western Blotting*. Esta enzima defosforilada ativa mecanismos envolvidos com a morte celular, sendo inibida por fosforilação. Nós encontramos que o tratamento agudo e crônico com 17β-estradiol aumentou a fosforilação de GSK-3β nas fatias controles e nas fatias submetidas a OGD. Esses resultados podem sugerir que esta é uma possível via pela qual o 17β-estradiol atua como agente neuroprotetor, uma vez que a fosforilação de GSK-3β inibe sua ação apoptótica.

Introdução

As doenças neurodegenerativas afetam um amplo espectro da população e, na maioria dos casos, levam à incapacidade física e/ou mental, envolvendo memória, cognição e linguagem. Dentre essas doenças do Sistema Nervoso Central podemos destacar as desordens cerebrovasculares que são uma das principais causas de morbidade e mortalidade entre adultos e idosos. Nos Estados Unidos, aproximadamente 600.000 casos são registrados por ano, onde foi estimado um custo total (direto e indireto) de 43 bilhões de dólares em 1998 [21]. Acredita-se que no Brasil a importância do problema seja da mesma magnitude.

A isquemia cerebral é caracterizada por uma redução severa ou por um bloqueio completo do fluxo sanguíneo ao cérebro. Ocorre em vários quadros clínicos, sendo o mais comum destes o "derrame" que é a interrupção focal do suprimento de sangue para uma parte do cérebro. Outro exemplo consiste na diminuição transitória do fluxo de sangue para o cérebro inteiro (isquemia global) como ocorre durante uma parada cardíaca [8].

Devido à própria natureza da injúria cerebral isquêmica a lesão resultante é produto da combinação de vários processos fisiopatológicos, que interagem de forma complexa. Os principais processos envolvidos são deficiência energética, perda da homeostase iônica celular, acidose, aumento nos níveis de cálcio intracelular e excitotoxidade mediada por radicais livres [7]. É principalmente durante o período de restabelecimento do fluxo sanguíneo à área afetada que ocorre a geração de grandes quantidades de radicais livre, os quais são espécies altamente reativas que promovem dano a lipídios, DNA, carboidratos e proteínas, contribuindo significativamente para a injúria neuronal [29].

Até o presente momento, não há qualquer protocolo terapêutico clinicamente eficaz para a prevenção e/ou tratamento das lesões isquêmicas.

Como modelo para o estudo da isquemia cerebral, uma alternativa para a experimentação animal *in vivo* pode ser o uso de sistemas *in vitro* como as culturas organotípicas de hipocampo de ratos expostas à privação de oxigênio e glicose (POG). Este modelo consiste na colocação das culturas hipocampais em presença de meio livre de glicose, dentro de uma incubadora com atmosfera anaeróbia [3,4]. Os modelos *in vitro* são simples de utilizar e reproduzem vários aspectos da isquemia *in vivo,* parecendo ser adequados para a análise de mecanismos envolvidos na morte celular isquêmica em geral, bem como para a investigação de drogas com potencial neuroprotetor. Em nosso laboratório este modelo vem sendo amplamente utilizado para avaliar o potencial de drogas neuroprotetoras, entre elas o lítio [5] e o extrato de Kava-kava (*Piper metisticum*) (manuscrito em preparação).

Os estrógenos são hormônios esteróides que exercem um largo espectro de efeitos por todo o organismo, incluindo o Sistema Nervoso Central. O potencial efeito neuroprotetor dos estrógenos tem ganhado crescente atenção nos últimos anos e vem sendo alvo de constantes estudos. Dados epidemiológicos demonstraram que fêmeas pré-menopausa exibem uma menor suscetibilidade ao dano causado pela isquemia do que machos e fêmeas pós-menopausa [14,26]. Isto pode ser devido ao fato de que fêmeas pré-menopausa possuem níveis mais altos de estrógenos circulantes, principalmente o 17β-estradiol. Além disso, o uso de estrógenos pela terapia de reposição hormonal parece diminuir o risco e a severidade de doenças neurodegenerativas como a doença de Alzheimer [37], Parkinson [28], Esquizofrenia [9] e parece melhorar a memória e a cognição [38].

Recentes estudos *in vivo* e *in vitro* têm mostrado que os estrógenos têm efeito neuroprotetor contra vários insultos tóxicos, incluindo excitotoxicidade, estresse oxidativo e toxicidade mediada pelo peptídeo β-amilóide [11], principal componente protéico das placas senis encontrada na doença de Alzheimer. Entretanto, o mecanismo da neuroproteção mediada pelo estradiol está apenas começando a ser elucidado [39].

O efeito neuroprotetor do estradiol pode ser mediado através de mecanismos dependentes ou independentes da ativação dos receptores estrogênicos α e β. Além disso, podem exercer efeitos a nível genômico e não genômico. Os receptores α e β são amplamente distribuídos no Sistema Nervoso Central e, quando ativados, podem levar a alteração da expressão de alguns genes, podendo aumentar a expressão de neurotrofinas e seus receptores e diminuir a expressão de receptores de glutamato [9]. Além disso, o estradiol pode ativar diretamente vias de sinalização sem o envolvimento dos receptores [9] e, entre outros, atuar como potente antioxidante diminuindo a peroxidação lipídica [22].

Outro mecanismo proposto para a neuroproteção exercida pelo estradiol é que ele é um potente vasodilatador, o que promove uma melhora na circulação sanguínea após um insulto isquêmico, levando a uma diminuição no tamanho da lesão [18].

Recentemente foi encontrado que tratamento de neurônios corticais com estradiol estimulam a fosforilação de Akt/PKB, um efetor imediatamente *downstream* da via PI-3K, protegendo as células da neurotoxicidade induzida por glutamato [17], sugerindo que a cascata da PI-3K pode estar envolvida na neuroproteção induzida por estradiol. Além disso, estradiol ativa a cascata das MAPK [4] que como a via da PI-3K, pode fosforilar e inativar proteínas promotoras da morte celular como a GSK-3β. O papel da GSK na indução da apoptose ainda não está bem elucidado [20]. Porém têm sugerido que a

ativação de GSK inibe importantes fatores de transcrição envolvidos na sobrevivência celular como fatores de choque térmico (HSF-1 – *Heat Shock Factor*) cuja ativação aumenta a expressão de proteínas de choque térmico (HSP – *Heat Shock Protein*) [15].

Em vista do que foi mencionado, este trabalho tem por objetivo avaliar o potencial efeito neuroprotetor do 17β-estradiol em fatias de cultura organotípica hipocampais de rato submetidas à privação de oxigênio e glicose bem como seu efeito na fosforilação de GSK-3β.

Neuroprotective effect of 17β-estradiol in organotypic slice cultures of rat hippocampus exposed to oxygen and glucose deprivation.

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Abstract

The brain is highly dependent on continuous blood flow for oxygen and glucose supply. Brain ischemia results in cellular degeneration and loss of function. Organotypic cultures are an *in vitro* model, which allows the study of the events and mechanisms underlying ischemic brain damage. Here we investigated the neuroprotective effect of 17β-estradiol in an *in vitro* model of ischemia. To mimic an ischemia, organotypic hippocampal slice cultures were exposed to oxygen and glucose deprivation (OGD), using an anaerobic chamber developed in our laboratory. Cellular death was quantified by measuring uptake of propidium iodide (PI). A decrease in incorporation of PI was observed in OGD slices both acute and chronic 17β-estradiol (10nM) treatment indicating a neuroprotective effect. To examine a possible mechanism by which estradiol prevents cellular death we evaluate the immunocontent of pGSK-3β by western blot analysis. This enzyme has been shown to trigger cellular death. We have found that acute and chronic treatment with 17β-estradiol increased the phosphorylation of GSK-3β in the control and OGD slices. These findings might suggest that this is a possible pathway by which 17β-estradiol acts as neuroprotective agent, once the phoshphorylation of GSK-3β inhibits its apoptotic action.

Theme: Disorder of the nervous system

Topic: Ischemia

Keywords: Cerebral ischemia, 17β-estradiol, Organotypic Culture, Hippocampus, GSK-3β, Neuroprotection.

1. Introduction

Neurodegenerative diseases affect a wide spectrum of the population. In the United States [36], about 600,000 people suffering a new or recurrent stroke each year, and in most cases lead to incapacity, involving memory, cognition, language and personality. The reduction in the supply of glucose and oxygen to the brain that occurs in cerebral ischemia leads to a complex cascade of cellular events that result in neuronal death [21,36].

In the course of the investigation of cerebral ischemia, an *in vitro* model, organotypic hippocampal slice culture is a valuable alternative to animal experiments. Organotypic cultures have been used to study mechanisms underlying neuronal death induced by hypoxia/aglycemia [25] and excitotoxins [27]. To model ischemic events, organotypic cultures are exposed to oxygen and glucose deprivation (OGD) using an anaerobic chamber. We have found that the response of organotypic cultures to lesion induced by OGD is quite similar to that shown by animals submitted to transient cerebral ischemia, suggesting the suitability of this model for the study of ischemic lesion and neuroprotective drugs [33].

Premenopausal females exhibit a lower susceptibility to stroke-related brain damage than males and postmenopausal females [14,26]. The purposed neuroprotection in premenopausal females may be related to higher levels of circulating estrogen principally 17β-estradiol. Furthermore, estrogen replacement therapy appears to decrease the risk and/or severity of neurodegenerative disease, such as Alzheimer's disease [37], Parkinson's disease [28], Schizophrenia [9], and may also improve memory and cognition [38].

The cellular mechanisms underlying the neuroprotective effects of estrogen are only beginning to be elucidated [39]. Estradiol might exert neuroprotective effects acting through estrogen receptor (named ER alpha and ER beta) dependent and ER independent, genomic and non-genomic means to attenuate neural injury [37]. In the different models used for investigation, it has been suggested that the genomic effect might be mediated by reduction of the number of glutamate receptors [10], altered synthesis of proteins directly involved in the initiation of apoptotic cascade [24,35] and the non-genomic effect by acting as potent antioxidant and inhibiting lipid peroxidation [22].

Furthermore estrogen may interact with intracellular signaling cascade [9] such as MAP kinase and PI-3 Kinase [12]. Interestingly, both MAPK and PI-3K pathway converge to inhibit GSK-3β [6,32] by phosphorylation. This enzyme has been shown to be one of the factors that trigger cellular death [16]. To explore this possible mechanism for the

neuroprotective effects of 17β-estradiol we tested its effect against lesion induced by the exposition of hippocampal organotypic slices cultures to oxygen and glucose deprivation (OGD) as well as its effect on GSK-3β phosphorylation.

2. Materials and Methods

2.1. Hippocampal-slice cultures

Organotypic hippocampal-slice cultures were prepared according to the method of Stoppini et al [30]. Briefly hippocampal slices were prepared from 6-8-day-old Wistar rats by removing the brain, dissecting hippocampi and making transverse slices (400μm) using a McIlwain tissue chopper. Slices were separated in a Hanks' balanced salt solution (HBSS) supplemented with 25-mM HEPES, 1% fungizone and 36 μl/100 ml garamicine, pH 7.2. Six slices were placed on a Millicell® -CM culture insert and then they were transferred to a six-well culture plate with 1 ml of culture medium consisting of 50% minimum essential medium (MEM), 25% horse serum and 25% HBSS, supplemented with (mM, final): glucose 36, glutamine 2, HEPES 25 and $NaHCO_3$ 4 (pH 7.3). Fungizone (1%) and garamicine (36 μl/100 ml) were added to the medium. The plates were then placed in an incubator at 37 ºC in an atmosphere of 5% CO_2. Medium was changed every 3 days. The slices were cultivated during 14 days. Pre-treatment was initiated 7 days after the beginning of the culture by adding 17β-estradiol (10 nM in DMSO) and this corresponds to chronic treated group (EC).

2.2. OGD

After 14 days *in vitro*, the cultures were exposed to OGD [19]. The induction of OGD was based on the method described by Strasser and Fisher [31], with some modifications [5]. Each insert was transferred to a sterilized plate rinsed twice with OGD medium composed of (mM): $CaCl_2$ 1.26; KCl 5.36; NaCl 136.89; KH_2PO_4 0.44; Na_2HPO_4 0.34; $MgCl_2$ 0.49; $MgSO_4$ 0.44; HEPES 25 (pH 7.2). They were then left in 1 mL of OGD medium for 15 min, after that the medium was exchanged for OGD medium previously bubbled with nitrogen for 30 min. Three inserts were processed in parallel, corresponding to three experimental groups: CE – chronic treatment group when 17β-estradiol was added at 7^{th} culture day, AE – Acute treatment group when 17β-estradiol was added in the

OGD medium, DMSO – The veichle (DMSO 0,01%) was added in the OGD medium. Subsequently the plate containing the inserts was transferred to an anaerobic chamber, which was closed, evacuated and nitrogen was rapidly injected. During the procedure the temperature was kept around 37 ºC. Cultures were maintained in these conditions for 60 min. After the OGD period, the cultures were removed from the chamber, the inserts were washed twice with HBSS and returned to the culture medium. 17β-estradiol was added in OGD medium and in culture medium during the 24-h recovery period.

2.3. Quantification of cellular death

Cellular damage was assessed by fluorescent image analysis of propidium iodide (PI) uptake [5,25]. After a 23-h recovery period, 7.5 μM PI was added to the cultures and incubated for 1 h. PI is excluded from healthy cells, but in cells that loose of the membrane integrity it enters cells, binds to DNA and becomes highly fluorescent. Cultures were observed with an inverted microscope (Nikon Eclipse TE 300) using a standard rhodamine filter set. Images were captured and then analyzed using the Scion Image software. The area where PI fluorescence was detectable above background was determined using the "density slice" option of Scion Image software and compared with the total CA1 area to obtain the percentage of damage [34].

2.4. Western blotting

After the fluorescent images were obtained the slices were lysed (SDS Stopping Solution 4% - SDS 4%, EDTA 2.1 mM, Tris 50mM). An aliquot was taken for protein determination and β-mercaptoethanol was added to a final concentration of 2% [33]. Samples were analyzed by minigel SDS-PAGE 12%. Thirty-five micrograms of protein from each sample were applied to the gels. After electrophoresis gels were blotted onto a nitrocellulose membrane using a semi-dry blotting system (Bio-Rad Trans-Blot SD). The membranes were blocked overnight with 5% powdered milk in tween-Tris-buffered saline (M-TTBS). Primary antibody was incubated overnight at 4ºC in M-TTBS (p-GSK-3β, 1:1000, Cell Signaling). After wash, the membranes were then incubated for 2 h with secondary antibody (anti-rabbit IgG peroxidase conjugated, 1:1000, Amersham). The chemiluminescence (ECL) was detected using X-ray films. The films were scanned and the percentage of band intensity was analyzed using OptiQuant software. For each

experiment, the test groups were referred to control non-treated cultures, which were considered 100%. .

2.5. Data analysis

Comparisons between experimental groups were by one-way ANOVA followed by Duncan's multiple range test. All data are presented as mean ± S.E.M.

3. Results

3.1 - Neuroprotective effect of 17β-estradiol

To examine the neuroprotective effect of 17β-estradiol we used organotypic slices cultures of rat hippocampus exposed to OGD for 60 min. Cellular death was quantified by measuring uptake of propidium iodide (PI). PI is a polar compound, which only enters cells with damage cell membranes. Inside the cell it binds to nucleic acids and becomes brightly red fluorescent, and has been used as an indicator of neuronal integrity and cell viability [6]. It was observed a significantly decrease in the incorporation of PI in the ischemic slices in both acute and chronic treatment with 17β-estradiol (10 nM) (71% and 78%, respectively) when compared with ischemic slices treated only with veichle (Fig. 1). These results indicate a neuroprotective effect of 17β-estradiol. In the control slices it was not detected difference between the treatments, indicating that there was a neither protective nor toxic effect.

3.2 – Effect of 17β-estradiol in GSK-3β phosphorylation

Because GSK-3β is the substrate of the PI-3K and MAPK pathways, both of which are actived in the presence of estrogen [12] we have determined if estrogen treatment has any effect on the phosphorylation state of GSK-3β since GSK-3β phosphorylated has its pro-apoptotic activity inhibited. For this, we used phosphospecific antibodies in a Western blotting procedure. It was observed that chronic treatment with 17β-estradiol increased phosphorylation of GSK-3β in the control and OGD exposed slices (28% and 23 % respectively) and acute treatment with 17β-estradiol also significatively increased phosphorylation of GSK-3β in control and OGD exposed slices (29% and 34%) when

compared with control slices with veichle. The OGD exposed slices treated with veichle were not different from control slices with veichle (Fig. 2).

4. Discussion

Over the past 100 years, the lifespan of women has increase to >80 years, consequently women are living an ever-increasing proportion of their lives in a hypoestrogenic postmenopausal state, which could contribute to an increased risk of cognitive dysfunction and a variety of neurodegenerative diseases [38]. Neurological diseases affect the qualities that make the lives of humans so special, and thereby a drug that could provide neuroprotection against neurodegenerative diseases such as hypoxic-ischemic damage, when found, would have a great clinical value.

Recently, it has been demonstrated, *in vitro*, that estrogen exerts neuroprotective effects against the neurotoxicity that results from serum deprivation [13], β-amyloid [1] and NMDA agonist treatment [2]. Then, 17β-estradiol has been most widely studied *in vivo* where has been showing neuroprotection effect in both sexes [18]

Although a number of studies have demonstrate the neuroprotective effect of 17β-estradiol its precise protection mechanism has not been elucidated [17].

In the present study, we have extended the previous findings by showing that 17β-estradiol has neuroprotective effect in organotypic cultures of hippocampus exposed to OGD. Our results demonstrate that 17β-estradiol treatment attenuate cellular death in this model. As indicated by PI incorporation analysis, not only chronic treatment but also acute treatment reduced in 78 and 71% respectively the size of lesion in OGD slices when compared with OGD slices with veichle (DMSO 0.01%). 17β-estradiol had no effect in control slices indicating that there were neither protective nor toxic effects, in this condition.

To examine a possible mechanism by which 17β-estradiol reduced cellular death we examined its effect on the phosphorylation of GSK-3β. Recently it has been shown that an inhibitor of this enzime reduced cell death of cortical neurons caused by PI-3K inhibition or serum withdrawal [33]. From the current literature it was demonstrated that estrogen stimulates PI-3K and MAPK pathways. Both of these pathways have been shown to be responsible for inactivate GSK-3β. In the present work we found that acute and chronic 17β-estradiol treatment increased the GSK-3β phophorylation in both control and OGD exposed slices. These results can suggest that this is a possible pathway by which 17β-

estradiol acts as neuroprotective agent, once the phoshphorylation of GSK-3β inhibits its apoptotic action.

These results are very important since the population ages and stroke is a leading cause of mortality and morbidity in the postmenopausal women. These data imply that older women may be benefit from the protective effects of estrogen replacement therapy that utilizes relatively low concentrations of hormone. The understanding of the mechanisms of estrogen-dependent neuroprotection might provide novel drug targets and lead to the generation of innovative therapeutic interventions for men and women.

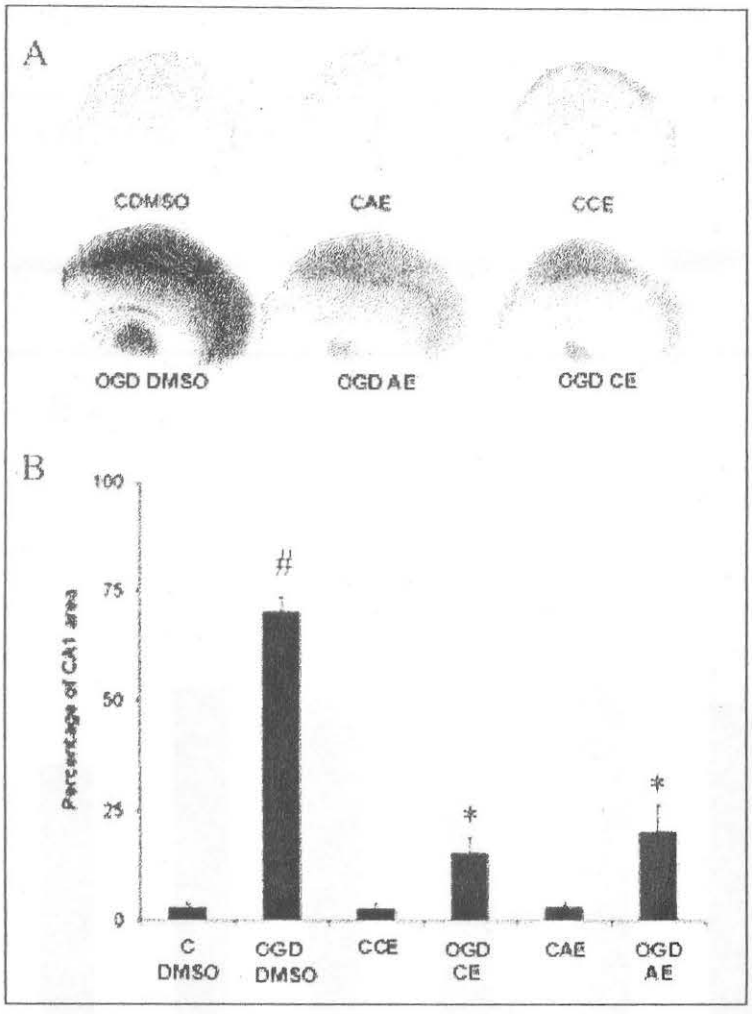

Figure 1 - Effect of 17β-estradiol on cellular damage induced by OGD for 60 min in organotypic cultures of hippocampus. (A) Representative images of cultures; (B) Quantitative analysis of incorporation of PI in CA1 area. C – control, OGD – slices exposed to oxygen and glucose deprivation, AE – Acute 17β-estradiol treatment, CE – Chronic 17β-estradiol treatment. * significantly different from OGD DMSO and controls slices. # significantly different from others groups. Bars represent S.E.M (n=15), $P< 0.05$. (one-way ANOVA and Duncan's test).

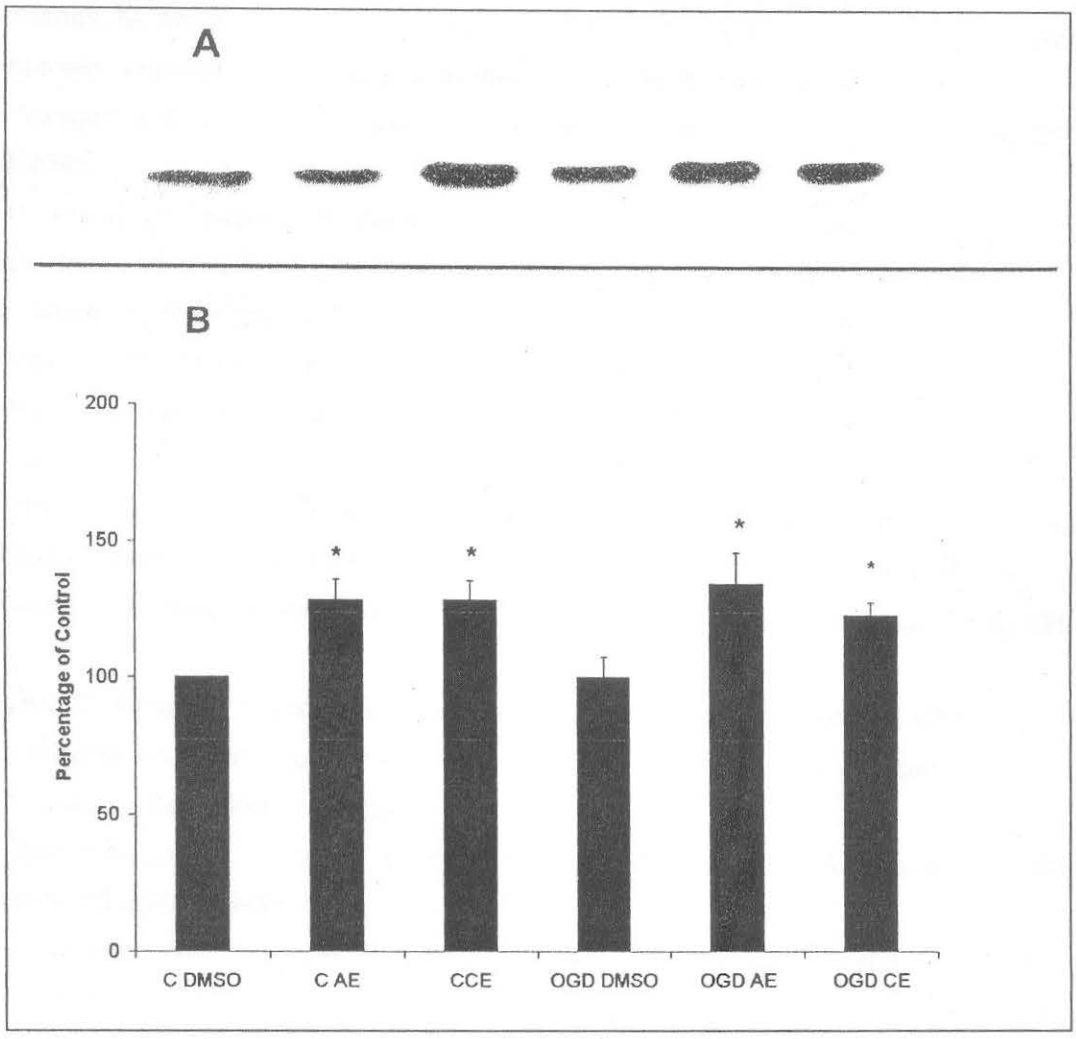

Figure 2 - Effect of 17β-estradiol on the porcentage of phosphorylated GSK-3β. A – Representative image of imunocontent of pGSK-3β. B – Quantitative analysis of imunocontent of pGSK-3β. Data are expressed as a percentage of veichle-treated controls not exposed to OGD (100%). C – control, OGD – slices exposed to oxygen and glucose deprivation, AE – Acute 17β-estradiol treatment, CE – Chronic 17β-estradiol treatment. * significantly different from control and OGD slices without treatmente with 17β-estradiol. Bars represent S.E.M (n=7) $P<0.05$. (one-way ANOVA and Duncan's test).

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