Anti-Angiogenic/Anxiolytic Effect of Chlordiazepoxide: Evidence on Dual Activity for Cancer Patients

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Abstract
Angiogenesis which is the recruitment of new blood vessels is a critical Process in several chronic diseases and is an essential component of tumor or cancer progression. Development of therapies aimed at inhibiting the angiogenesis, in combination with classical anti-cancer therapies, is among the most intensively studied approaches to treat the cancer. In this study the effect of chlordiazepoxide (which is a proper anxiolytic drug) on angiogenesis in microcarrier-based collagen gel was assayed. Chlordiazepoxide beside its anxiolytic effect in patients showed strong inhibitory effect on human umbilical vein endothelial cells (HUVECs) tubulogenesis (angiogenesis) in collagen matrix, without toxic effects in the studied doses.

Keywords: Chlordiazepoxide, Anti-angiogenic, Anxiolytic, HUVECs

Introduction
Angiogenesis is the formation of new capillary from pre-existing vessels and plays a critical role in physiological process such as development and wound healing,(1) and pathologic processes.(2, 3) Many studies demonstrated that angiogenesis is regulated by the balanced between a variety of positive and negative factors. Thus, decrease in activity of angiogenic factors and increase of anti-angiogenic factors may provide a proper approach to for managing various cancers. The anti-angiogenic therapy strategy is involves suppression of angiogenesis factors and promotion of anti-angiogenic factors. Since the identification of endostatin as an inhibitor of angiogenesis,(4) a variety of anti-angiogenic compounds both synetic and natrual were tested for their anti-angiogenic properties. Drug development has become a promising strategy for identification of new anti-angiogenic and anti-tumor components, there are also some drugs that we use them as a treatment of other disease that have a dual activity such as anti-angiogenic activity, beside their main functions. Chlordiazepoxide potentially has significant health-promoting effects, including amnestic,(5) anxiolytic,(6) hypnotic, skeletal muscle relaxant properties and anticonvulsant.(7) Half life of chlordiazepoxide is medium to long (5– 30 hours) but its active metabolite has a very long half life. It was reported that ulcers and dermatologic problems, both of which involve emotional factors, were reduced by chlordiazepoxide. Chlordiazepoxide also indicated as a treatment for the management of acute alcohol withdrawal syndrome.(8) Chlordiazepoxide was the first benzodiazepine to be synthesized and the discovery of chlordiazepoxide was by pure chance. Benzodiazepines require special precaution if used in the elderly, pregnancy, children, alcohol- or drug-dependent individuals and individuals with comorbid psychiatric disorders. Benzodiazepines impair learning and memory via their action on benzodiazepine receptors which causes a dysfunction in the cholinergic neuronal system in mice.(9)
Previously it has been shown that the most profound reduction in the turnover of 5HT (serotonin) in rats was found to caused by chlordiazepoxide then the other benzodiazepines. Serotonin which is closely involved in regulating mood, may be one of the causes of feelings of depression in rats using chlordiazepoxide or other benzodiazepines.(10) Chlordiazepoxide also

decreases prolactin release in rats.(11) Inhibition of acetylcholine release in mouse hippocampal synaptosomes in vivo by chlordiazepoxide was showed.(12) This has been found by measuring sodium-dependent high affinity choline uptake in vitro after pretreatment of the mice in vivo with chlordiazepoxide. This may play a role in chlordiazepoxide's anticonvulsant properties.(12) Differential regulation of the behavioral effects of chlordiazepoxide,(13) and Psychotherapeutic effect was considered too.(14)

Although there are many study concerning different properties of chlordiazepoxide, its properties and its effect on angiogenesis are unclear. The aim of our study is to investigate the anti-angiogenic effects of chlordiazepoxide on HUVECs model. Chlordiazepoxide is an important drug because it uses regularly for patient with stress and anxious, as it has anxiolytic effect.

Material and Methods

Drug prepration: Pure chlordiazepoxide was obtained from pharmacology department of Medical university of Kermanshah and authenticated by this department.

Culture of HUVEC: The purchased HUVECs were exited from frozen stock and grown on tissue culture-treated plastic in Dulbecco's modified eagle's medium (DMEM) containing 10% heat-inactivated fetal bovine serum (FBS), and then were centrifuged for 5 min at 1000g. The pellet was washed for an additional time and re-suspended in DMEM containing 10% fetal bovine serum (FBS), supplemented with 100U/ml penicillin and 100µg/ml streptomycin in 24 well culture plates and maintained at 37°C with 5% CO2 until 90% confluent. After cell count and determining cell viability, the suspension was transferred into appropriate cell culture flasks and the culture medium was added. HUVECs between passages 2 and 6 were used in our cell culture experiments.

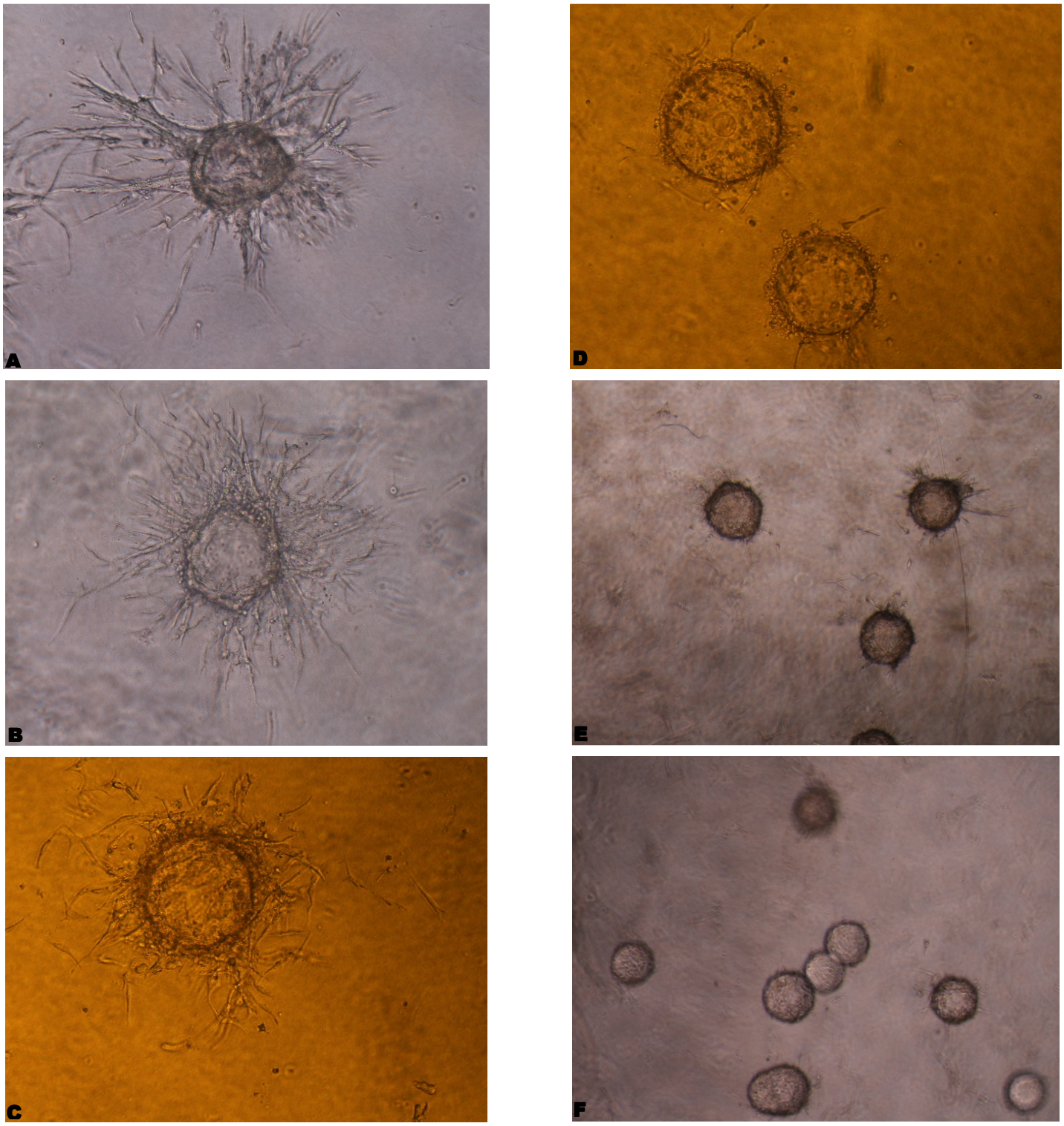
Cytotoxicity assay: To determining maximum non-toxic and cytotoxic concentrations of chlordiazepoxide, several concentrations were prepared and added to medium containing confluent HUVEC cell line. After 72 hours of incubation, culture medium was centrifuged to obtain cell-free medium and then the cell viability was determined by Lactate dehydrogenase (LDH) assays. Cytotoxicity assay was assessed by colorimetric measurement of LDH release according to LDH kit protocol. Cells were cultured in 96-wells culture plates at a density of 1×10^4 cells/well in DMEM supplemented with 2% FBS and incubation at 37ºC and 5% CO2 for 24 hr. Thereafter, different concentrations of chlordiazepoxide were added to the wells and the plates were incubated for additional 72 hr. 100µl of supernatant of wells were transferred to new 96-wells culture plate and LDH release assessed by LDH Kit at 490 nm with background subtraction at 630 nm.

Preparation of collagen gels: For collagen gel formation, 7 volumes of cold type I collagen solution with 1 volume of 10X minimal essential medium and 2 volume of sodium bicarbonate solution (11.76 mg/ml) were mixed in a sterile flask kept on ice to prevent immediate gelation.

HUVEC capillary tube formation in collagen matrix and evaluation of angiogenesis in vitro: HUVECs were grown in DMEM supplemented with 10% FBS at 37°C and 5% CO2 and after 2-6 passages were used for this experiment. Then cells were mixed with sterilized cytodex-3 microcarriers coated beads with gelatin at a ratio of 30 cells per bead in 1 ml of DMEM medium supplemented with 10% heat-inactivated FBS. The mixture was shaken gently every 20 minutes for 4 hr at 37°C and 5% CO2. Thereafter, it was transferred to a 24-well tissue culture plate and left for 12- 16 hours in 1 ml of DMEM at 37°C and 5% CO2. The following day, beads with cells were re-suspended in type 1 collagen gel and 50 µl of collagen/bead mixture was added to each well of a 96-well tissue culture plate and allowed to clot for 20 min at 37°C, 5% CO2. Then, 250 µl of DMEM medium was added to each well. In order to study anti-angiogenic effect of chlordiazepoxide, different concentrations of the extract (0, 10, 40, 80, 120, and 150µg/ml) were added to the wells. After 3–5 days of treatment, the anti- tubulogenesis effects of hydroalcholic extract of oak were monitored microscopically. All the angiogenic cells and capillary-like structures then photographed with a digital camera.

Results

Effects of the chlordiazepoxide at different doses on angiogenesis were shown in Fig. 1(A–F). Part A shows angiogenesis of the untreated endothelial cells (negative control). Endothelial cells attached to particles have been proliferated and migrated through the collagen matrix. B, C, D, E and F: angiogenesis of the endothelial cells treated with pure chlordiazepoxide at 0, 10, 40, 80, 120, and 150 µg/ml respectively. Clearly we can see



tubulogenesis of the endothelial cells through the collagen matrix were inhibited completely in 120 µg/ml, and also in 80 µg/ml we have about 60 percent inhibition. The LDH release from Chlordiazepoxide-treated cells wasn't increased with compared to untreated control cells confirming the specific inhibitory Chlordiazepoxide on angiogenesis without affecting cell viability.

Discussion

Most of the tested doses inhibited blood vessel formation (tubulogenesis), but had no toxic effect on human umbilical vein endothelial cells in any of the tested concentrations (this was confirmed by Cytotoxicity assay). 80 µg/ml concentration would be safer to use in cancer patients because of probable side effect which may be caused by higher chlordiazepoxide concentrations. Therefore, as these properties and result showed, chlordiazepoxide can be used as a specific anti-angiogenesis/anxiolytic drug beside the classical anti- cancer therapies in cancer patients. As most of the patients with tumor are nervous and anxious (because of the disease psychological pressure)

using chlordiazepoxide not only psychologically help them to be relaxed but also because of anti-angiogenic effect it may help to prevent cancer progression and metastasis. Although more invivo and invitro studies in animal models and lab base angiogenic systems are needed to clarify the curative and preventive role of chlordiazepoxide in cancer onset and progression

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