

The Effects of TGF- β 3, 17- β Estradiol and Bisphenol A on Osteoprotegerin Production in Osteoblasts

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ABSTRACT

Bone metabolism is a complicated and challenging issue in both physiological and pathological states. The homeostasis of the tissue is majorly being managed by the competing activities of osteoblasts and osteoclasts. Osteoprotegerin (OPG) is a decoy receptor for RANKL and it inhibits osteoclast differentiation by binding RANKL and being produced primarily by osteoblasts. The decrease in OPG level causes excessive osteoclast activation which results in high bone resorption that overcomes new bone formation. Therefore, it is important to understand the mechanism of OPG production and identify its regulators. In this study, it was aimed to address the effects of TGF- β 3, 17- β estradiol and bisphenol A (BPA), an endocrine disrupter, on OPG production from osteoblasts. For this purpose, hfOB cells were treated with TGF- β 3, 17- β estradiol and BPA for 48 hours both alone and in combinations. The effects of these agents were evaluated by sandwich-ELISA. The analysis showed that TGF- β 3 and 17- β estradiol treatment causes an increase in OPG levels when used in combination. It was also discovered that BPA exhibits antagonistic effect on OPG production when used along with TGF- β 3 and 17- β estradiol.

Keywords: 17- β Estradiol, Bisphenol A, Osteoprotegerin, TGF- β 3.

TGF- β 3, 17- β Estradiol ve Bisphenol A'nın Osteoblastlar Üzerinde Osteoprotegerin Üretimine Etkileri

ÖZ

Kemik metabolizması hem fizyolojik hem de patolojik durumlarda karmaşık ve zorlu bir konudur. Dokunun homeostazisi büyük oranda osteoblastların ve osteoklastların karşılıklı aktiviteleri ile idare edilmektedir. Osteoprotegerin (OPG), başlıca osteoblastlar tarafından üretilen ve RANKL ile bağlanarak osteoklast farklılaşmasının inhibe edilmesini sağlayan bir tuzak reseptördür. OPG seviyesindeki düşüş yoğun osteoklast aktivasyonuna ve dolayısı ile yeni kemik yapımını aşan miktarda kemik yıkımına sebep olmaktadır. Bu nedenle OPG üretim mekanizmasını anlamak ve OPG'nin düzenleyicilerini tanımlamak önem arz etmektedir. Bu çalışmada TGF- β 3, 17- β estradiol ve bir endokrin bozucu olan bisfenol A'nın (BPA) osteoblastlardan OPG üretimine etkilerinin belirlenmesi amaçlandı. Bu amaçla hfOB hücrelerine TGF- β 3, 17- β estradiol ve BPA 48 saat boyunca tek başlarına ve kombinasyon halinde uygulandı. Bu faktörlerin etkileri sandviç ELISA yöntemi ile değerlendirildi. Yapılan analizde TGF- β 3 ve 17- β estradiolün kombinasyon halinde kullanımının OPG seviyelerinde artış sağladığı belirlendi. Ayrıca, çalışmada BPA'nın OPG üretimi üzerine antagonistik etkilerini TGF- β 3 ve 17- β estradiol ile birlikte uygulandığında gösterdiği gözlemlendi.

Anahtar Kelimeler: 17- β Estradiol, Bisfenol A, Osteoprotegerin, TGF- β 3.

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INTRODUCTION

Bone is a complex tissue which comprises both organic and inorganic compounds. Thus, the phenomenon of bone homeostasis is matching with the complexity of the tissue itself as many influencing factors such as hormones, growth factors, vitamins, minerals and various other proteins get involved with the process (Bouillon and Suda 2014, Rodan 1998). Osteoblasts and osteoclasts stand as two frontiers for the homeostasis of the tissue as they are chief responsible for bone remodeling events. The homeostasis process heavily depends on the equilibrium between the actions of these two cell types. Osteoblasts are responsible for new bone formation, while osteoclasts oppose this action by resorbing the bone tissue and by these battling events, the renewal of the adult skeleton goes on throughout the entire lifetime of an individual (Langdahl et al. 2016).

Osteoblasts are specialized cells which are producing severely important bone-specific proteins such as osteocalcin, osteopontin, bone sialoprotein, osteoprotegerin (OPG), osteonectin, etc. and originate from the undifferentiated mesenchymal stem cells of bone marrow (Katagiri and Takahashi 2002, Schoppet et al. 2002). On the other hand, osteoclasts are multinucleated bone resorbing cells and their precursors are of monocyte/macrophage family that located within bone marrow (Udagawa et al. 1990). The osteoclastogenesis has a complex stimulation system as there are multiple factors both trying to enhance and inhibit the differentiation at the same time. The main actors of osteoclast differentiation are RANK, RANKL and OPG proteins which interact with each other and regulate osteoclastogenesis in bone. The osteoclast precursor cells have a specific surface receptor called RANK which binds with a tumor necrosis factor (TNF) superfamily protein called RANKL to induce osteoclast differentiation (Lam et al. 2001). The RANKL is being secreted by many cell types including osteoblasts and lymphoid tissue cells; but the protein is primarily produced by B and T-lymphocytes and the production of RANKL by these cells reported to be up regulated in some bone tissue disorders (Chaparro et al. 2020, Kawai et al. 2006, Yeo et al. 2011). However, OPG; a TNF receptor superfamily protein which is a soluble decoy receptor secreted by osteoblasts, binds with RANKL and blocks RANK/RANKL complex formation for inhibiting osteoclast differentiation (Dougall 2010, Simonet et al. 1997, Tong et al. 2019). There is a matter of balance in RANKL/OPG ratio in normal physiology but in certain situations like osteoporosis, the balance is disturbed in favor of RANKL and massive amounts of bone loss occurs as the OPG levels decrease (McClung 2007). Along with osteoporosis, OPG is also thought to has a significant role in bone regeneration, especially in cases of bone

fractures (Köttstorfer et al. 2014). TGF- β superfamily proteins and steroid hormones are known to have a great importance in pathways related with bone tissue. Alongside with their numerous roles, estradiol and TGF- β superfamily proteins also act as positive influencers for OPG production (Murakami et al. 1998, Thirunavukkarasu et al. 2001, Zuo et al. 2020). Although, TGF- β 1 of the TGF superfamily was studied for its effect on OPG, TGF- β 3 remains relatively understudied.

Bisphenol A (BPA) (2,2-bis (hydroxyphenyl) propane), is a widely used plasticizer and a known endocrine disrupter which counteracts estrogens by binding to estrogen receptors especially α & β (Diel et al. 2000, Vitku-Kubatova et al. 2018). BPA is reported to have negative effects on many organs and tissue types including brain (Özden Akkaya et al. 2018), skeletal muscle (Jing et al. 2019) and bone (Thent et al. 2018). BPA is also shown to have inhibitory effects on expressions of bone related markers *in vitro*. It has been reported that 24 hours of BPA exposure cause a decrease in the expressions of RANK, RUNX2 and Osterix along with decreased ALP activity (Hwang et al. 2013). Alongside with these findings, BPA causes lower actin density in cytoskeletal structures of osteoblasts and also significantly down regulates some of the important osteoblast proliferation and differentiation markers (Thent et al. 2020). Although, BPA is known to disturb bone metabolism, its effects on OPG production are still undetermined.

In this study, it was aimed to demonstrate the effects of TGF- β 3 17- β estradiol and BPA on OPG production from osteoblasts both alone and in certain combinations *in vitro*.

MATERIALS and METHODS

Cell Culture

Human fetal osteoblastic cells (hfOB) (ATCC 1.19 CRL-11372, passage 7) were cultured in DMEM/F12 (with 15 mM HEPES) (Biowest, France) with 1% penicillin-streptomycin (10.000 U/mL) (Gibco, UK), 10% fetal bovine serum (Biowest, South America Origin), 200 mM L-glutamin (Gibco, UK) at 37°C in 5% CO₂ environment. The culture medium was changed every three day intervals. Cells were trypsinized (0.25 % trypsin and 0.01% EDTA) (Capricorn, Germany) at 70-80% confluence and passaged.

Cell Culture Treatments

hfOB cells were seeded in normal hfOB growth medium at a density of 1x10⁶ cells / flask in 75 cm² culture flasks after passaging. After 24 hours, culture medium was replaced with the desired culture media respective to the experimental groups. The design of the experimental groups are given in table 1. A control group was set using standard hfOB growth medium. In the first experimental group, the hfOB

medium was supplemented with 10 ng / mL TGF- β 3 (Peprotech, USA). In the second group, cells were cultured with 10 nM 17- β estradiol (BioGems, USA) in hfOB medium. The culture media for the third group consisted of hfOB medium supplemented with 10 ng / mL TGF- β 3 and 10 nM 17- β estradiol together. Then, in the fourth group, 12.5 μ g/mL bisphenol A (Sigma-Aldrich, USA) was added to the hfOB medium and finally, for the fifth group, 12.5

μ g/mL bisphenol A was added into 10 ng/mL TGF- β 3 and 10 nM 17- β estradiol supplemented hfOB medium. Cells were incubated at standard conditions for 48 hours. After 48 hours, the supernatant was collected into centrifuge tubes. The collected samples were centrifuged at 1000 g for 10 minutes to avoid cellular debris. After filtering the supernatant using 0.22 μ PES syringe filters, samples were stored at -80°C until further use.

Tablo 1. Deneý grupları ve kùltür medyumunda yapılan uygulamalar.

Table 1. Experimental groups and respective treatments in culture mediums.

Groups	Treatments
Control Group	hfOB growth medium
Group 1	10 ng/mL TGF- β 3 supplemented medium
Group 2	10 nM 17- β Estradiol supplemented medium
Group 3	10 ng/mL TGF- β 3 + 10 nM 17- β Estradio supplemented medium
Group 4	12.5 μ g/mL BPA supplemented supplemented medium
Group 5	10 ng/mL TGF- β 3 + 10 nM 17- β Estradiol + 12.5 μ g/mL BPA supplemented supplemented medium

Sandwich ELISA

The measurement of OPG levels in cell culture supernatant samples were determined by using a commercial ELISA kit (Abcam, ab100617) as per instructions of the manufacturer. The sensitivity of the assay was 1 pg / mL and the range was 1.23 pg / ml to 900 pg / mL. Standard solutions were run in duplicates while the samples were run in triplicates. Six samples were used for each group and the absorbance was measured at 450 nm. The standard curve and the sample measurements were made using CurveExpert 1.3 software.

Statistics

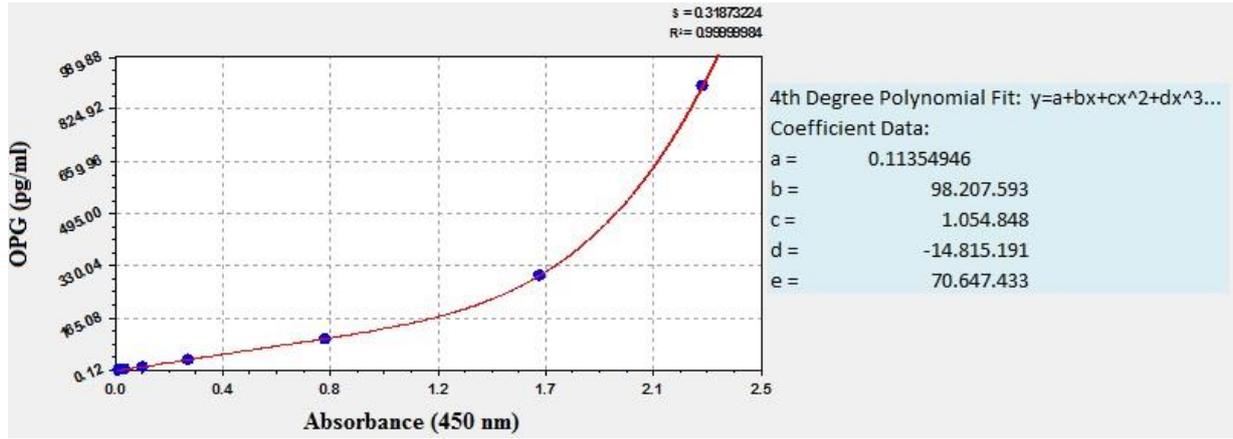
The data was analyzed for outliers using Tukey's Fence Test and the normal distribution of the samples were examined with Shapiro-Wilk Test. As there were no outliers found and the data was found to be normally distributed, One-Way ANOVA with Tukey's multiple comparisons test was used to assess the data. All statistical analysis were performed with SPSS v20 (IBM) and the graphs were made using GraphPad Prism 8 software.

RESULTS

For all experimental groups, no visible changes in the morphology of cells were observed during culture. The OPG level measurements of the groups were determined according to a 4th degree polynomial fit standard curve ($R^2=0,99999984$) (Fig. 1). The

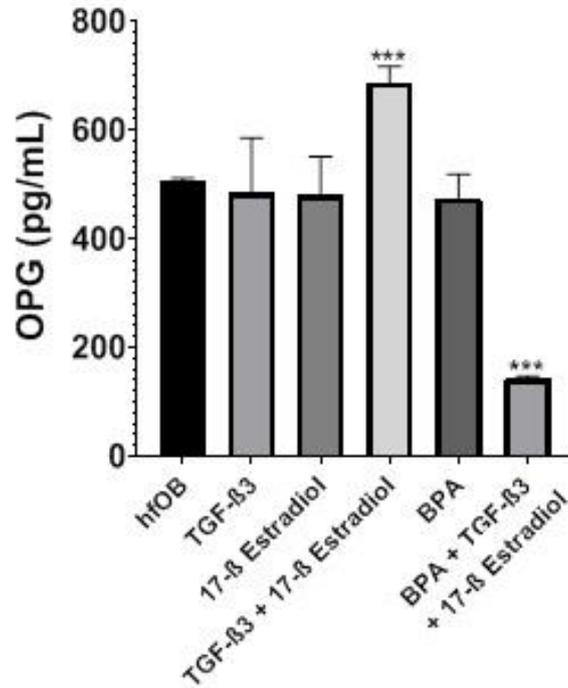
calculated data were assessed statistically by using One-Way ANOVA (Summary given in Table 2) and the groups were compared with each other using Tukey's Multiple Comparisons Test (Table 3). The OPG levels in control group were found to be approximately 507 ($\pm 2,07$) pg/mL in standard culture conditions. Statistical analysis showed that there were no significant changes in the OPG levels for TGF- β 3 or 17- β estradiol treated samples when compared to the control samples (p : 0,986 and 0,966) as the OPG levels were measured as 485 ($\pm 41,1$) pg/mL and 480 ($\pm 28,9$) pg/ml respectively. However, the combination of TGF- β 3 and 17- β estradiol resulted with the highest OPG levels in culture samples as much as 687 ($\pm 12,3$) pg/mL concentration which was significantly higher ($p < 0,001$) when compared to the control group and all other experimental groups (Table 2; Figure 2).

BPA, on the other hand was found to have no significant effects on OPG secretion when supplemented into normal growth medium alone (p : 0,926). The average concentration of OPG was found to be 474 ($\pm 17,9$) pg /mL in BPA treatment group. However; when BPA was used in combination with TGF- β 3 and 17- β estradiol, the OPG levels decreased drastically as only 143 ($\pm 1,68$) pg /mL OPG was measured which was significantly lower when compared to the control ($p < 0,001$) and all other experimental groups (Table 2; Figure 2).



Şekil 1: 4. derece polinomial fit standart eğrisi ve denklemini ($R^2=0,9999984$).

Figure 1: 4th degree polynomial fit standard curve and it's equation ($R^2=0,9999984$).



Şekil 2: Deneş gruplarında OPG seviyeleri. TGF- β 3 ve estradiol ilave edilmiş grup en yüksek OPG salgılayan grup olarak belirlenirken aynı gruba BPA ilavesi en düşük OPG seviyesiyle sonuçlandı. Dięer guruplar arasında belirgin bir fark gözlenmedi.

Figure 2: OPG levels among experimental groups. TGF- β 3 and Estradiol supplemented group observed as the highest OPG releasing group while BPA supplementation in the same group showed the lowest OPG levels. There were no significant difference was visible between other groups.

Tablo 2. One-Way ANOVA test özeti.

Table 2. Summary of One-Way ANOVA test.

ANOVA summary	
F	59,89
P value	<0,001
P value summary	***
Significant diff. among means	Yes
R square	0,9117

Table 3. Deneý gruplarında OPG seviyeleri Tukey's Multiple Comparisons Test ile karşılaştırıldı (***: p<0,001).
Table 3. Comparison of OPG levels among experimental groups were assessed with Tukey's Multiple Comparisons Test (***: p<0,001).

Compared Groups	Significancy	p value
hfOB vs. TGF- β 3	Ns	0,986
hfOB vs. Estradiol	Ns	0,966
hfOB vs. TGF- β 3+Estradiol	***	<0,001
hfOB vs. BPA	Ns	0,926
hfOB vs. BPA+TGF- β 3+Estradiol	***	<0,001
TGF- β 3 vs. Estradiol	Ns	>,999
TGF- β 3 vs. TGF- β 3+Estradiol	***	<0,001
TGF- β 3 vs. BPA	Ns	>,999
TGF- β 3 vs. BPA+TGF- β 3+Estradiol	***	<0,001
Estradiol vs. TGF + Est	***	<0,001
Estradiol vs. BPA	Ns	>0,999
Estradiol vs. BPA+TGF- β 3+Estradiol	***	<0,001
TGF- β 3+Estradiol vs. BPA	***	<0,001
TGF- β 3+Estradiol vs. BPA+TGF- β 3+Estradiol	***	<0,001
BPA vs. BPA+TGF- β 3+Estradiol	***	<0,001

DISCUSSION

In the study it was aimed to investigate the effects of TGF- β 3, 17- β estradiol and BPA on OPG production from osteoblasts both alone and in various combinations *in vitro*. The reduced amounts of OPG production in certain bone degenerative diseases like osteoporosis and a search for better bone regeneration in cases like bone fractures prompted scientists to understand the secretion mechanism of OPG to utilize it as a tool against osteoclastic activity. Therefore, many scientists attempted to identify positive and negative regulators of OPG. Along with a variety of proteins like interleukin 1 α , bone morphogenic protein 2, TNF α , etc.; especially estrogen and TGF- β had been identified as important positive regulators of OPG (Hofbauer et al. 1999, Hofbauer et al. 1998, Murakami et al. 1998). In the case of TGF- β superfamily, although there are some reports indicating the effects of TGF- β 1 (Murakami et al. 1998), the effect of TGF- β 3 on OPG levels had not been investigated individually. As TGF- β 1 and β 3 target same pathways and share similar actions on osteogenesis (Grafe et al. 2018), a similarity is thought to be possible in the context of OPG production as well.

The doses and concentrations of TGF- β 3 and 17- β estradiol were determined from their known effective dose for osteogenic differentiation of cells (Hong et al. 2006, Karbanodva et al. 2010). While, the dose of BPA was decided upon a previous study on hfOB cells which IC₅₀ value of BPA was reported as 12.5 μ g/mL (Thent et al. 2020).

The results of the study indicate that both 17- β estradiol and TGF- β 3 did not improve OPG levels in hfOB samples after 48 hours of culture when they supplemented alone. However, a combination of both, resulted in increased levels of OPG. The results of the studies conducted by Hofbauer et al. (1999) and Saika et al. (2001) demonstrate that estrogen successfully stimulates the OPG expression in human osteoblasts and mouse stromal cells, alone. In contrast with the findings of these reports, no individual up regulation was seen in OPG levels when 17- β estradiol was used alone. The cell line hfOB, had been shown to express both 46 kDa and 77 kDa isoforms of estrogen receptor (ER) α in western blot analysis in previous studies (Foo et al. 2007). However, both Hofbauer et al. (1999) and Saika et al. (2001) further transfected their cells with ER α gene. Therefore, the results of the recent study may indicate that hfOB cells do not contain sufficient amounts of ER α to be further stimulated by estradiol.

hfOB cells are osteoblastic cells which spontaneously differentiates into mature osteoblasts in culture conditions as they showed increasing levels of osteogenic markers in normal culture conditions and share some similarities with bone marrow mesenchymal stem cells as they express some mesenchymal markers in early stages (Yen et al. 2007). Wang et al. (2006) verified that the ER α expression is being increased during the process of osteogenic differentiation. Thent et al. (2020) also reported positive expressions for all estrogen receptors; ER α , ER β and estrogen related receptor

gamma, in hfOB cells after using a 6 day differentiation protocol.

TGF- β 3 is known to have a key role in early osteoblast differentiation and RUNX2 expression which is essential for osteoblast differentiation as well (Grafe et al. 2018). In this study, supplementation of TGF- β 3 may induce maturation of hfOB cells and increase the expression of ER α during this process. That probability may explain the increased levels of OPG when TGF- β 3 and 17- β estradiol used in a combination, since it is known that ER α transfected hfOB reported to have increased OPG mRNA levels by the stimulation of 17- β estradiol alone (Hofbauer et al. 1999). The synergetic effects of TGF- β 3 and 17- β estradiol in the context of OPG production may be explained by the increased levels of ER α under the influence of TGF- β 3 which causes 17- β estradiol to be more effective.

The BPA experiments also support this view as there was no effect seen on OPG levels when BPA was used alone but a significant decrease was visible when it was supplemented with TGF- β 3 and 17- β estradiol. Routledge et al. (2000), reported that BPA has lower binding affinity to estrogen receptors α and β compared to 17- β estradiol. Hiroi et al. (1999) also reported that BPA has lower affinity to estrogen receptors but despite this much lower affinity, they reported that BPA can compete around half of the estrogenic activity in same concentration. Hiroi et al. (1999) also investigated the effects of BPA and 17- β estradiol together and they discovered that BPA is having antagonistic effects only when it binds with ER α in the presence of 17- β estradiol.

The recent study suggests that it is possible for TGF- β 3 to cause an increase in ER α levels on hfOB cells by inducing their maturation and as a result 17- β estradiol and BPA have a chance to reveal their effects on OPG production through ER α . To back this view, an experiment group should be established containing a combined supplementation of BPA and TGF- β 3 and ER α levels should be examined throughout the study by comparing the measurements between all experimental groups in future studies.

CONCLUSIONS

The study indicates that TGF- β 3 and 17- β estradiol have an enhancing effect on OPG secretion from osteoblasts and this effect may be correlated with ER α levels of the cells which should be further investigated. Although, it was known that BPA had various effects on bone metabolism, its effects on OPG was demonstrated for the first time. But in order to identify how this action was induced by BPA, further research is needed especially by demonstrating whether its effect is through ER α and if there is any correlation with the expression levels of the receptor.

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Ethical Statement: This study is not subject to the permission of HADYEK in accordance with Article 8 (k) of the "Regulation on Working Procedures and Principles of Animal Experiments Ethics Committees".

Conflict of Interest: Author declares no conflict of interest for this manuscript.

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