

The Effects of Tamoxifen and Its Metabolites on
Platelet Function and Release of Reactive Oxygen Intermediates

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ABSTRACT

Tamoxifen is effective in the prevention and treatment of breast cancer but its use is associated with an increased risk of thrombosis. The mechanism for this effect is unknown. Reactive oxygen intermediates enhance platelet-dependent thrombosis and, in oncological studies, tamoxifen has been shown to increase production of reactive oxygen species. Therefore, the effects of tamoxifen and its bioactive metabolites on platelet activity and platelet reactive oxygen species were determined. Platelets were incubated with tamoxifen or the metabolites 4-hydroxy-tamoxifen (4-OH), N-desmethyl tamoxifen (NDM), or 4-hydroxy-N-desmethyl tamoxifen (endoxifen). Tamoxifen metabolites have been previously shown to possess enhanced bioactivity and, consistent with this observation, tamoxifen metabolites but not tamoxifen modestly increased platelet aggregation. These effects were similar with platelets isolated from male or female subjects. Platelet nitric oxide (NO) release or cGMP levels were not altered by incubation with tamoxifen or any of its metabolites. Incubation with tamoxifen metabolites increased stimulation-dependent platelet superoxide release (8.1 ± 1.6 arbitrary units (a.u.) for control vs. 15.2 ± 3.5 a.u. for 4-OH; $P < 0.01$). Co-incubation with a superoxide dismutase mimetic eliminated the tamoxifen metabolite-induced enhancement of platelet aggregation. Corresponding to increased superoxide release, incubation with tamoxifen metabolites enhanced the functional activation of NADPH oxidase as determined by phosphorylation of its subunits p47^{phox} and p67^{phox}. In summary, incubation of platelets with the active metabolites of tamoxifen increases stimulation-dependent superoxide release through a

NADPH oxidase-dependent mechanism. This results in modest changes in platelet function and appears to be consistent with previous oncological studies demonstrating tamoxifen-dependent increase in reactive oxygen species generation.

Tamoxifen, the progenitor of the selective estrogen receptor modulator class of drugs (SERMS) is widely used for the prevention and treatment of breast cancer, however, a number of studies have shown adverse effects.(Pagano et al., 2001) Tamoxifen induces apoptosis in breast cancer cells through caspase-3 and JNK1 pathways, which are initiated at the cell membrane via oxidative mechanisms. These effects are thought to be related to oxygen radical overproduction occurring during tamoxifen metabolic activation.(Mandlekar et al., 2000)

In the breast, tamoxifen is an estrogen antagonist, but its use is also associated with pro-estrogenic effects in other tissues including increases in hepatic coagulation factor synthesis.(Chang et al., 1996; Pritchard et al., 1996) Consistent with this finding, tamoxifen use is also associated with a significantly increased risk of venous thrombosis.(Duggan et al., 2003) To date, the only serious adverse event clearly associated with the SERM raloxifene is a tripling of the risk of venous thromboembolic events.(Cummings et al., 1999) This observation is important as the large clinical trial Raloxifene Use for The Heart (RUTH) is underway to study whether this SERM reduces the risk of coronary heart disease in women at high risk.(Walsh et al., 1998) As acute coronary syndromes are primarily caused by platelet-dependent aggregation, determining if SERMS potentially alter platelet function and contribute to the process of thrombosis would be of relevance to clinical studies. Although the effects of SERMS on platelet-dependent thrombosis are not known, tamoxifen metabolites have been investigated in other vascular biological systems. They have been

shown to inhibit vascular smooth muscle proliferation in an injury model and may impact lipid metabolism.(Yue et al., 2000)

Because tamoxifen's receptor dependent and independent estrogenic effects may be mediated by its active metabolites,(Jordan et al., 1977; Crewe et al., 1997; Stearns et al., 2003) studies were conducted to determine whether tamoxifen or its principal metabolites might influence platelet activity or alter platelet generation of reactive oxygen intermediates, specifically superoxide and nitric oxide (NO), known to modulate platelet function. In these studies, the effects of tamoxifen and N-desmethyl tamoxifen (NDM) were initially examined as these species have the highest concentrations in plasma. In addition the effect of 4-hydroxy-tamoxifen (4-OH) and 4-hydroxy-N-desmethyl-tamoxifen (endoxifen) were also studied as these species have approximately one hundred fold greater potency as estrogen receptor antagonists as compared to tamoxifen.(Furr and Jordan, 1984; Stearns et al., 2003)

METHODS

Chemicals and Reagents

Prostaglandin E₁ (PGE₁) lucigenin, sodium citrate, NaH₂ PO₄, ADP, and dextrose were purchased from Sigma (St. Louis, MO). Fibrinogen was purchased from Enzyme Research Laboratories (South Bend, IN). Tamoxifen, N-desmethyl tamoxifen (NDM) and z-4-hydroxy-tamoxifen (endoxifen) were obtained from SIGMA (ST. LOUIS, MO) and 4-hydroxy-n-desmthyl-tamoxifen (4-OH) was synthesized by Dr. Ross Weatherman, Department of Medicinal Chemistry, Purdue University, as described in detail elsewhere.(Stearns et al., 2003)

Preparation of Platelets

Blood samples, using acid/citrate/dextrose (ACD) as anticoagulant (1:9 v/v), were obtained from healthy male (n=8) and female (n=12, premenapausal) volunteers who denied having taken any drugs, medications, hormonal treatment, or supplements that could alter platelet function in the two weeks before blood sampling. No subjects had hypertension, hyperlipidemia, diabetes, or currently smoked. Platelet rich plasma (PRP) was obtained after centrifugation at 150 x g, 20 minutes, 24°C.

Physiologic concentrations of tamoxifen and its metabolites were chosen to be studied. The supernatant (platelet rich plasma: PRP) was separated and

incubated with total concentrations of tamoxifen and its metabolites, chosen to be in the range that we have observed to be present in plasma when tamoxifen is prescribed to women at the usual dose of 20 mg per day. (Lee et al., 2003; Stearns et al., 2003) This approach assumes that free and bound concentrations of drugs are similar in plasma and PRP. Tamoxifen (1 μ M) or the tamoxifen metabolites N-desmethyltamoxifen (NDM, 1 μ M) or 4-hydroxytamoxifen (4-OH, 10 nM) or endoxifen (100 nM) were incubated with PRP for 30 min at 24°C. Some of the platelets were subjected to incubation with 17- β -estradiol (0.1-10 μ M) for relative comparison or vehicle control. After the addition of the equal volume of platelet washing buffer (sodium citrate 10 mM, NaCl 150 mM, EDTA 1mM, dextrose 1% pH 7.4 with prostaglandin in a 1:10.000 ratio) the PRP was centrifuged at 350 x g for 15 minutes, 24°C. The platelet pellet was resuspended in Tyrode's solution consisting of the following components: NaCl 140 mM, KCl 6 mM, Mg₂SO₄ 2mM, NaHPO₄ 2 mM, Hepes 6 mM (pH 7.4). Platelet counts were determined using a Coulter Counter, model ZM (Coulter Electronics, Miami, FL). For the NO measurements and aggregation studies the platelet counts were then adjusted to final concentration of 2.5 x 10⁵ platelets/ μ L. For the superoxide reaction, 3.5-4 x 10⁵ platelets/ μ L was used.

Measurement of Platelet Nitric Oxide Production and Aggregation

We adapted a NO-selective (Freedman and Keaney, 1999) micro-electrode (Inter Medical Co., Ltd., Nagoya, Japan) for use in a standard platelet

aggregometer (Payton Associates, Buffalo, N.Y.) in order to monitor platelet NO production and aggregation simultaneously, as previously described.(Freedman and Keaney, 1999) Platelet NO production was rigorously quantified as the integrated area under the curve of the signal detected by the micro-electrode following platelet activation with 20 μ M thrombin receptor-activator peptide (TRAP). As compared to measuring peak height, this approach allows enhanced quantification and comparison of relative signal. Aggregation of washed platelets was monitored using a standard nephelometric technique as previously described in response to TRAP, ADP (2 μ M), or collagen (2mg/l) and % aggregation was determined 3 minutes after the addition of the agonist.(Freedman and Keaney, 1999)

Measurement of Platelet Superoxide Production

Lucigenin-derived chemiluminescence was used to estimate aggregation-dependent superoxide production from stimulated platelets using a lumiaggregometer (whole blood lumiaggregometer, model 500-CA, Chronolog Corp., Havertown, PA) as previously detailed.(Freedman and Keaney, 1999) Washed platelets (4×10^5 platelets/ μ L), preincubated with lucigenin (250 μ M) in HEPES buffer for 3 minutes, were placed into the lumiaggregometer (aggregometer with luminescence detection). After stabilization of the signal, phorbol myristate acetate (PMA) (0.6 μ M)-stimulated superoxide production and aggregation were simultaneously measured while stirring at 1000 rpm at 37°C.

Measurement of Platelet cGMP

Briefly, washed platelets were mixed with 10^{-5} M volume of 3-isobutyl-methyl-xanthine (IBMX). Trichloroacetic acid (final concentration, 5% vol/vol) was added. The supernatant was extracted, acetylated, and assayed for cGMP by an ELISA methodology using cGMP antiserum as previously described.(Clutton et al., 2004)

Phosphorylation of Platelet Proteins

Washed platelets, incubated with [32 P]orthophosphate (1 mCi/mL), were passed through PD-10 columns to remove free [32 P]orthophosphate as previously described.(Clutton et al., 2004) Platelet aggregation was stopped by the addition of lysis buffer. In other experiments, the lysate was centrifuged (15,000 g, 5 min, 4°C) to separate the Triton X-100-soluble and -insoluble cytoskeletal (CSK) fractions. The CSK fraction was resuspended in LIPA buffer. The proteins were resolved on SDS-polyacrylamide gels, transferred to polyvinylidene difluoride membranes, and immunoblotted with the specified primary polyclonal antibody. Immunoblots were probed with species-specific secondary antibodies coupled to horseradish peroxidase and visualized with chemiluminescent substrate.(Clutton et al., 2004) Autoradiography was used to determine phosphorylation of [32 P]-labeled proteins. The resulting

autoradiographs were analyzed by quantitative two-dimensional densitometry. Scanning densitometry was performed using NIH Image for data analysis.

Statistical Analysis

Differences between groups were determined using an unpaired Student's *t* test. The effects of interventions were analyzed using a paired *t* test. A statistically significant difference was assumed with a value of $P < 0.05$. All data are expressed as the mean \pm SEM.

RESULTS

To determine the effect of physiological concentrations of tamoxifen and its metabolites on platelet aggregation, platelets were incubated with 1 μ M tamoxifen, 100nM 4OH, 1 μ M NDM, 100nM endoxifen, 10 μ M 17- β -estradiol, or vehicle control at room temperature in the absence of light exposure for 30 minutes. Incubated platelets were then stimulated with 20 μ M TRAP and the extent of platelet aggregation was measured in a platelet aggregometer (Figure 1). There was a significant increase in platelet aggregation after incubation with the tamoxifen metabolites NDM, 4-OH, or endoxifen. Platelets were also stimulated with either ADP or collagen after incubation with increasing concentrations of tamoxifen, 4OH, NDM, endoxifen, or 17- β -estradiol. Incubation with ADP led to similar results with statistically significant increase in aggregation for all three metabolites ($n=3$, $P=0.04$, Figure 2). Although there was a trend towards increased collagen-induced aggregation after incubation with the three metabolites, this difference was not statistically significant ($n=3$, $P=0.1$, data not shown). There was no statistically significant change in platelet aggregation with tamoxifen using any of the platelet agonists. There was also an increase in aggregation after incubation with the superpharmacological level of 17- β -estradiol (Figures 1 and 2). Incubation with 1 μ M 17- β -estradiol did not significantly alter platelet aggregation (Figure 2).

To determine the effect of tamoxifen and its metabolites on platelet release of reactive oxygen intermediates, the effect of tamoxifen and its metabolites on platelet NO production was studied. Platelets were incubated with 1 μ M tamoxifen, 100nM 4OH, 1 μ M NDM, 100nM endoxifen, or 10 μ M 17- β -estradiol. Platelet NO release was not significantly altered after incubation with 17- β -estradiol, tamoxifen, or any of its metabolites (Figure 3). Increased NO is reflected by an accumulation of cGMP as a direct result of the activation of soluble guanylate cyclase. Incubation with tamoxifen or its metabolites did not significantly alter platelet cGMP levels in TRAP stimulated platelets (Figure 3). Thus, cGMP levels mirrors the lack of change in the platelet-derived NO as a result of incubation with tamoxifen or its metabolites.

While platelet NO levels minimally alter platelet aggregation,(Freedman et al., 1997) platelet function is known to be enhanced by the reactive oxygen species superoxide. Therefore, the effect of tamoxifen and its metabolites on platelet superoxide release was examined. Platelets were incubated with 1 μ M tamoxifen, 100nM 4OH, 1 μ M NDM, 100nM endoxifen, 10 μ M 17- β -estradiol, or vehicle control. Incubated platelets were then stimulated with 600nM PMA. Superoxide release was measured in a lumiaggregometer (Figures 4 and 5a). By this method, there was no significant change in superoxide release with tamoxifen and a statistically significant increase in superoxide release after incubation with NDM, endoxifen and 4-OH (n=3; * P <0.05). Platelet superoxide release was almost eliminated after incubation with 10 μ M 17- β -estradiol but

incubation with 1 μ M 17- β -estradiol failed to have a significant effect. To confirm that the mild enhancement of platelet aggregation was due to the increase in superoxide release, the effect of the tamoxifen metabolites endoxifen and 4-OH on platelet aggregation and superoxide release were repeated after coincubation with the membrane permeable superoxide dismutase (SOD) mimetic tempol (1mM). Tempol significantly decreased TRAP-stimulated platelet aggregation, restoring it to near baseline (Figure 5b). In addition, treatment with tempol also decreased platelet superoxide release from 15.1 ± 2.9 to 3.3 ± 1.1 a.u. for 4-OH ($n=4$, $P<0.01$ as compared to control). Findings were similar for endoxifen (data not shown). No change in platelet aggregation was found for tamoxifen after co-incubation with tempol ($n=4$, $P=ns$).

Initially identified in phagocytic cells, NADPH oxidase has since been identified as the primary producer of superoxide in vascular tissues. To determine the role of NADPH oxidase in the change in tamoxifen-dependent release of superoxide, the CSK fractions isolated from TRAP-stimulated platelets were immunoblotted with antibodies to p67-phox and p47-phox. Western blot analysis revealed that 3 minutes after TRAP-stimulation there was a significant increase in the amount of p67-phox present in the CSK of platelets that had been incubated with 4-OH or endoxifen (Figure 6). In addition, incubation with NDM, 4-OH, or endoxifen led to enhanced p47-phox after TRAP stimulation. However, there was not a significant increase in either p47- or p67-phox present in the CSK fraction isolated from tamoxifen-treated platelets 3 minutes after TRAP

stimulation (Figure 6). Incubation with 17- β -estradiol did not alter levels of either p47- or p67-phox (data not shown).

DISCUSSION

Relatively little is known about the mechanism of direct effects of tamoxifen or its metabolites on platelets. The presence of the androgen receptor (Nealen et al., 2001) and both the ER α and the ER β forms of the estrogen receptor have been shown to be present in human platelets. (Khetawat et al., 2000; Nealen et al., 2001; Jayachandran and Miller, 2003) However, the biochemical mechanisms involved in estrogen receptor-mediated signaling in platelets remain unclear. In patients with breast cancer, an antiestrogen regimen has been shown to not change platelet active prostacyclin or thromboxane. (Marttunen et al., 1996) Despite the clear association with enhanced venous thrombosis, (Duggan et al., 2003) some studies have failed to demonstrate changes in coagulation dependent proteins known to be associated with such a risk. (Love et al., 1992; Mannucci et al., 1996) This has included a lack of significant change in the markers of activated coagulation or fibrinolysis (fibrinopeptide A, prothrombin fragment 1 + 2, thrombin-antithrombin complex, D-dimer), (Mannucci et al., 1996) and a paradoxical decrease in fibrinogen levels. (Love et al., 1992) A previous study that examined platelets from women on tamoxifen exhibited decreased platelet adherence to endothelial cell matrix as compared to platelets from premenopausal women. (Miller et al., 1994) The differences between this study and our findings may be due to the specific patient populations (both pre- and post-menopausal) compared in the previous study (Miller et al., 1994) or to the distinctly different mechanisms regulating platelet adhesion vs. platelet

aggregation. However, in this study, *in vitro* tamoxifen incubation did not alter platelet adhesion and metabolites were not studied. These authors later demonstrate that tamoxifen-incubated platelets have enhanced adhesion to collagen,(Miller et al., 1995) leaving the effect of tamoxifen on platelet adhesion unresolved.

Although coagulation cascade dependent proteins are normally considered when evaluating venous thromboembolism, platelets can contribute to both venous and arterial thrombosis. Once activated, platelets further stimulate thrombus formation and recruit additional platelets to the growing thrombus.(Santos et al., 1991) Platelet activation and recruitment are tightly regulated by products of the endothelium including prostacyclin and NO. Exogenous NO inhibits platelet adhesion and aggregation, and prevents thrombosis.(de Graaf et al., 1992; Freedman et al., 1997) While dramatic changes in platelet redox status occur during normal aggregation, conditions that provoke oxidative stress without inducing a florid aggregation response have also been shown to be prothrombotic.(Freedman et al., 1996) Reactive oxygen intermediates contribute causally to many pathophysiologic conditions, and superoxide, in particular, is known to enhance the platelet aggregation response.(Handin et al., 1987)

In the current study, tamoxifen metabolites but not tamoxifen modestly enhanced platelet function as measured by an increase in platelet aggregation.

This effect did not appear to be due to altered platelet NO release as confirmed by both electrochemical detection and platelet cGMP levels. However, a statistically significant increase in superoxide release was observed with the tamoxifen metabolites and this change normalized when a SOD mimetic was added suggesting that change in platelet aggregation was due to the production of superoxide. Interestingly, tamoxifen, by initially partitioning into the membranes in breast cancer cells, has been shown to induce generation of transmembrane signals and an oxidative stress.(Gundimeda et al., 1996)

It was notable that these effects occurred with the metabolites and not tamoxifen itself. Tamoxifen metabolism is complex and involves a number of cytochrome P450 isoforms that catalyze conversion of the parent drug to NDM via cytochrome P450 3A, to endoxifen via the genetically polymorphic CYP2D6 and to 4-OH via a number of different enzymes. NDM is the tamoxifen metabolite that is present in the highest concentrations in plasma (Lee et al., 2003) and our previous data suggest formation of this metabolite is mediated by cytochrome P450 3A4/5.(Stearns et al., 2003) Although not tested in this study, variation in metabolism of these metabolites might contribute to individual risk of thrombosis.

To further understand the potential of direct receptor-mediated effects, estrogen incubation was also analyzed. Estrogen has been suggested to inhibit platelet aggregation through activation of eNOS,(Nakano et al., 1998) and a

number of studies suggest a gender effect on eNOS activity. However, we saw no difference in effect comparing platelets from either male or female donors. Although we observed no significant increase in platelet NO after incubation with 17- β -estradiol, we utilized a NO selective electrode and cGMP levels while the findings of the previous study were inferred by indirect measurements analyzing nitrate/nitrite. It is important to note that the proaggregatory changes we saw with 17- β -estradiol occurred after incubation with superpharmacological levels of estrogen and physiological and pharmacological levels had no effect in our studies. However, the doses of tamoxifen used were within pharmacological range (Decensi et al., 1999) and the concentrations of metabolites were also chosen to be in the range that we have observed to be present in plasma when tamoxifen is prescribed to women at the normal dose of 20 mg per day. (Lee et al., 2003; Stearns et al., 2003)

Previously, tamoxifen has been shown to induce apoptosis through elevation of intracellular calcium in HepG2 human hepatoblastoma cells. The role of reactive oxygen species in tamoxifen-induced apoptosis was studied in hepatoblastoma cells and caused a sustained increase in intracellular reactive oxygen species. Inhibitors of NAD(P)H oxidase significantly blocked the tamoxifen-induced generation of reactive oxygen species. (Lee et al., 2000) In phagocytotic cells, superoxide is generated via a one-electron reduction of molecular oxygen by the multicomponent NADPH oxidase. The NADPH oxidase complex consists of the membrane-bound cytochrome b_{558} (composed of the two subunits gp91-phox and p22-phox) and the cytosolic proteins, p47-phox, p67-

phox. Activation of this highly regulated enzyme involves the phosphorylation and translocation of the cytosolic components to the membrane-bound cytochrome b_{558} , where the catalytically functional oxidase is assembled. NADPH oxidase has also been identified in vascular cells and, recently, specific subunits have been demonstrated in platelets. (Krotz et al., 2002; Clutton et al., 2004) In the platelet, collagen activation induces NAD(P)H oxidase-dependent superoxide release, which in turn enhances availability of released ADP, resulting in increased platelet recruitment. Previous studies have demonstrated the expression of the NAD(P)H oxidase subunits p47- and p67-phox. (Krotz et al., 2002) These findings are consistent with our observation that the tamoxifen metabolites, to a varying degree, alter expression of either p47- and/or p67-phox. Although a previous study showed that incubation of cultured endothelial cells with 17- β -estradiol decreased expression of the NADPH oxidase subunit gp9-phox and this effect was prevented by coincubation with the estrogen receptor antagonists tamoxifen, (Wagner et al., 2001) this subunit has not been identified in platelets and estrogen did not alter either p47- and p67-phox.

In summary, tamoxifen is effective in the prevention and treatment of breast cancer but its use is associated with an increased risk of thrombosis. The mechanism for this effect is unknown. As reactive oxygen intermediates enhance platelet-dependent thrombosis, the effects of tamoxifen and its bioactive metabolites on platelet activity and platelet reactive oxygen species were studied. These studies showed that incubation of platelets with the active metabolites of

tamoxifen increases stimulation-dependent superoxide release through a NADPH oxidase-dependent mechanism. This results in enhanced platelet function and appears to be consistent with previous oncological studies demonstrating tamoxifen-dependent increase in reactive oxygen species generation.

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FIGURE LEGENDS

Figure 1. Representative tracings of the effect of 17- β estradiol, tamoxifen, or tamoxifen metabolites on platelet aggregation. Aggregation was induced with 20 μ M TRAP and monitored in a standard aggregometer as change in light transmittance over time.

Figure 2. Effect of 17- β estradiol, tamoxifen, or tamoxifen metabolites on platelet aggregation. Platelets were incubated in the presence or absence of increasing concentrations of tamoxifen or its metabolites or incubation with 17- β estradiol or vehicle control at 37°C for 20 minutes. Incubated platelets were stimulated with 2 μ M ADP and extent of platelet aggregation was measured in a platelet aggregometer (n=5; * P ≤0.05 compared to control without estrogen).

Figure 3. The effect of tamoxifen, or tamoxifen metabolites, or 17- β estradiol, on NO production and cGMP levels. Platelets were incubated with 1 μ M tamoxifen, 10nM 4OH, 1 μ M NDM, 100nM endoxifen, 10 μ M 17- β estradiol, or vehicle control at 37°C for 20 minutes. Incubated platelets were stimulated with 20 μ M TRAP and platelet-dependent NO release was measured using an electrochemical detector (n=5; P =ns compared to control). For cGMP levels, IBMX-treated platelets were stimulated with 20 μ M TRAP after a 20-min incubation with tamoxifen, tamoxifen metabolites, estrogen, or vehicle control. cGMP was measured in in platelets in which the reaction was stopped 3 min after TRAP-

stimulation. The cGMP concentration is expressed as the mean \pm SEM for three experiments (P =ns compared to control).

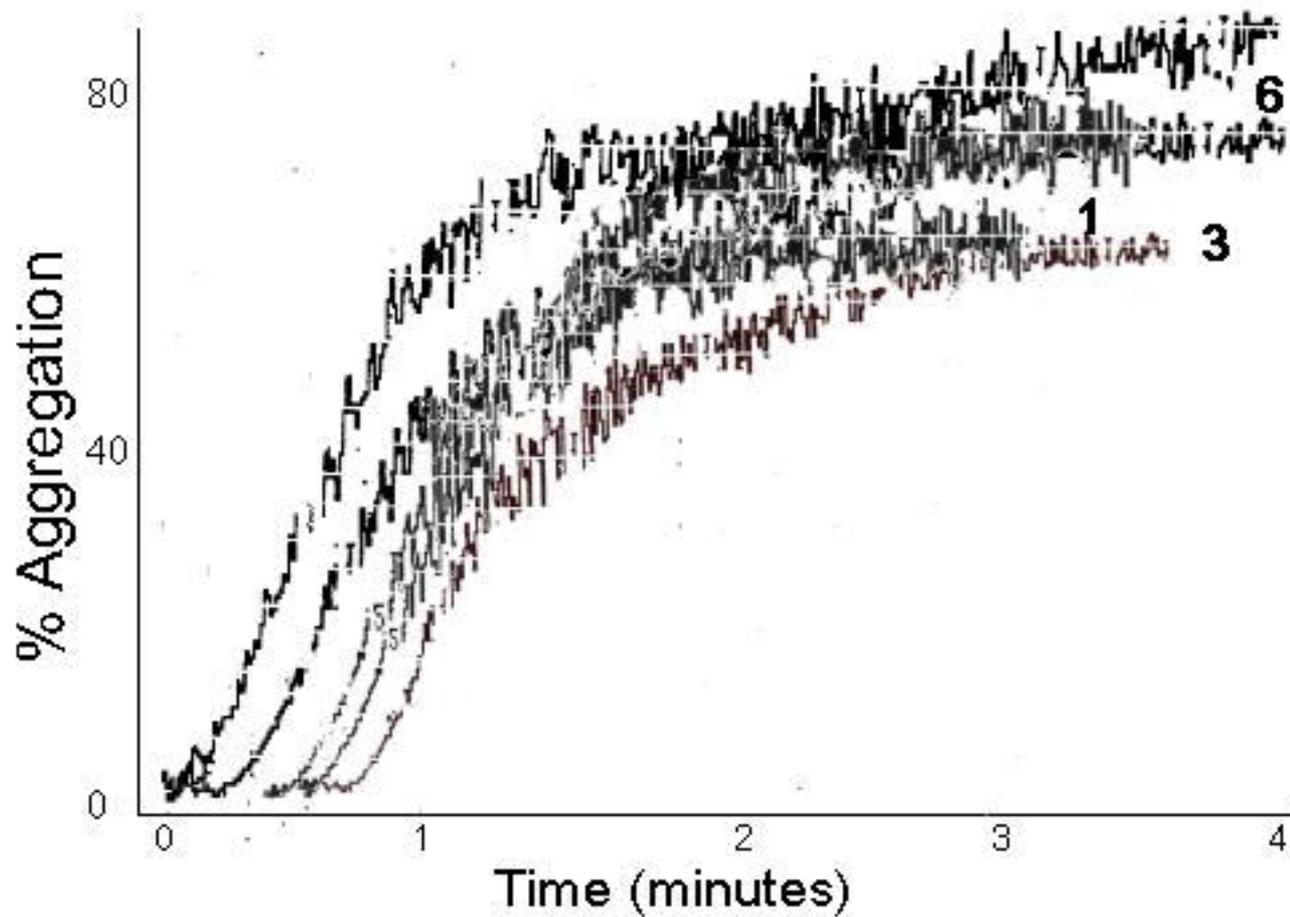
Figure 4. Representative tracings of the effect of 17- β estradiol, tamoxifen, or tamoxifen metabolites on platelet superoxide release (arb. Units on Y axis, time on x axis).

Figure 5a. Effect of tamoxifen, tamoxifen metabolites, or 17- β estradiol on platelet superoxide release. Platelets were incubated with 1 μ M tamoxifen, 100nM 4OH, 1 μ M NDM, 100nM endoxifen, 10 μ M 17- β estradiol or vehicle control at room temperature in the absence of light exposure 30 min. Incubated platelets were then stimulated with 600nM PMA. Superoxide release was measured in a lumiaggregometer (n=3; * P <0.05 compared to control; ** P <0.01 compared to control).

Figure 5b. Effect of superoxide inhibition on changes in platelet aggregation induced by tamoxifen, tamoxifen metabolites, or 17- β estradiol. Platelets were incubated in the presence or absence of tamoxifen or its metabolites or incubation with 10 μ M 17- β estradiol or vehicle control at 37°C for 20 minutes. These experiments were repeated in the presence of Tempol. Incubated platelets were stimulated with 20 μ M TRAP and extent of platelet aggregation was measured in a platelet aggregometer (n=4; * P ≤0.05 compared to control without Tempol).

Figure 6. Effect of tamoxifen and its metabolites on translocation of p47- and p67-phox to the platelet cytoskeleton. Isolated platelets were preincubated with 1 μ M tamoxifen, 100nM 4OH, 1 μ M NDM, 100nM endoxifen, or vehicle control for 20 min and stimulated with 25 μ M TRAP. The Triton X-100-insoluble fraction was analyzed by Western blot using antibodies specific for p47- and p67-phox. Densitometry was performed to quantify the amount of p47- and p67-phox protein present and expressed as the mean \pm SEM (n=3, * P <0.05 compared to control).

Figure 1



1= Control; 2= 17- β estradiol ; 3=tamoxifen; 4= NDM;
5 = 4-OH; 6=Endoxifen

Figure 2

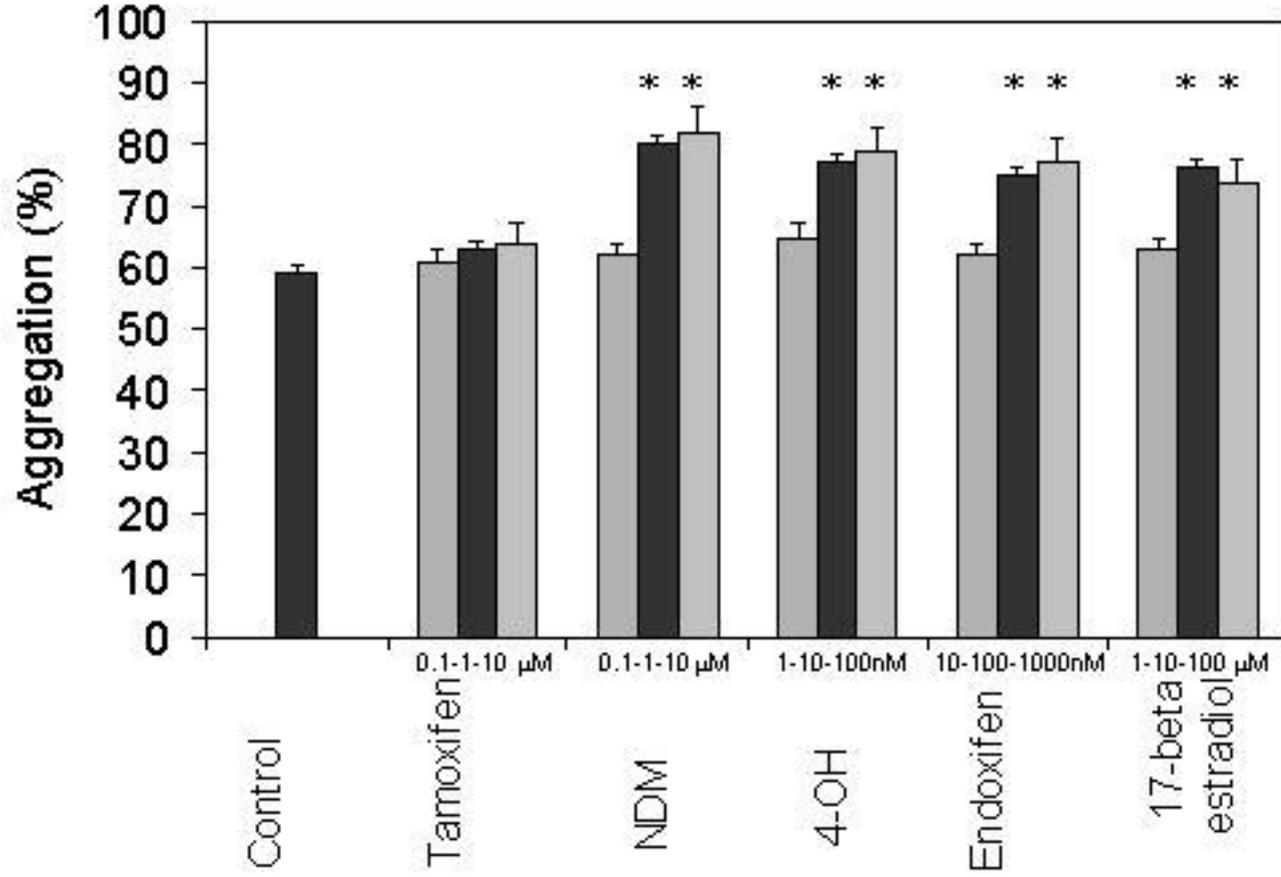


Figure 3

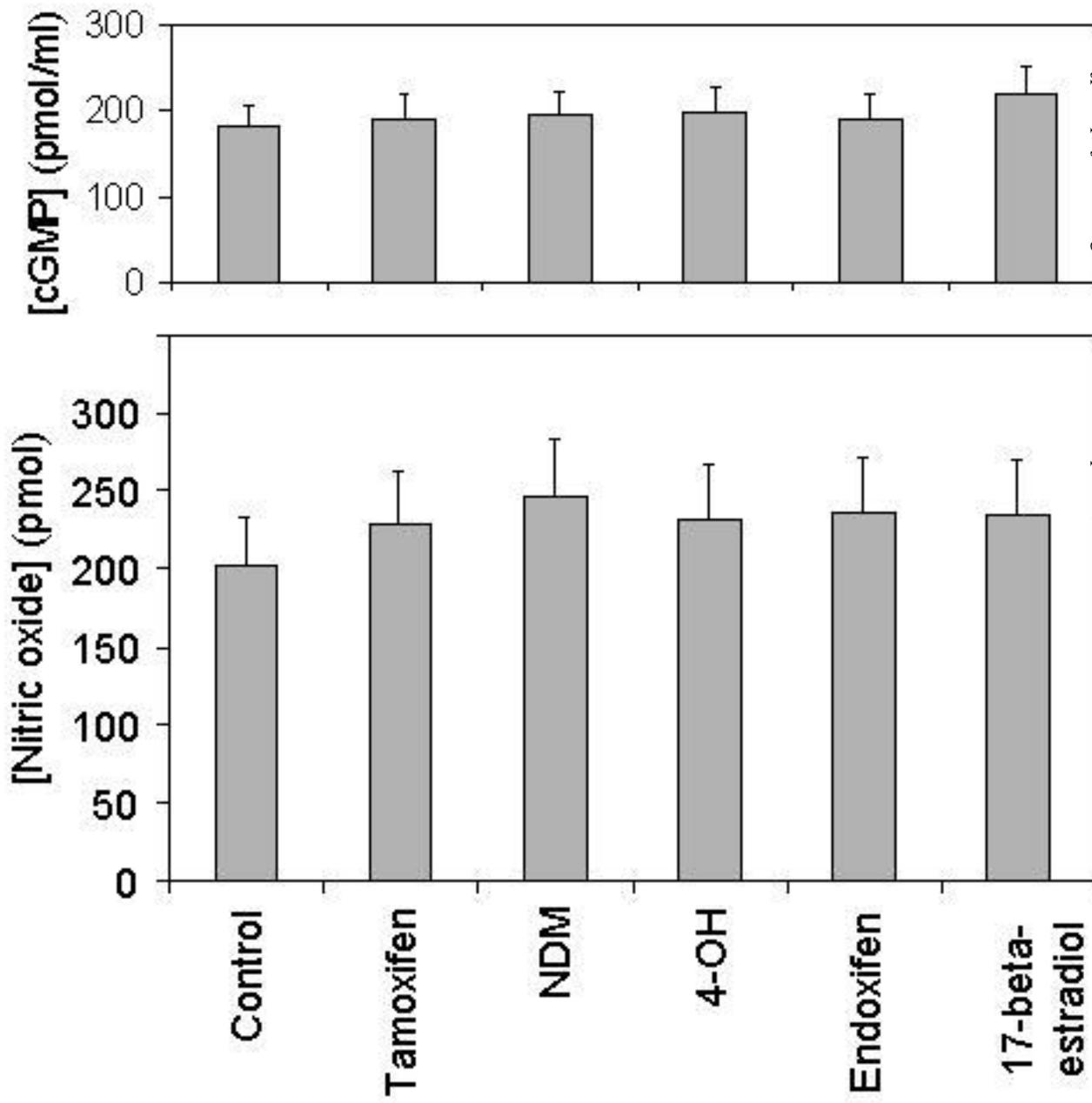
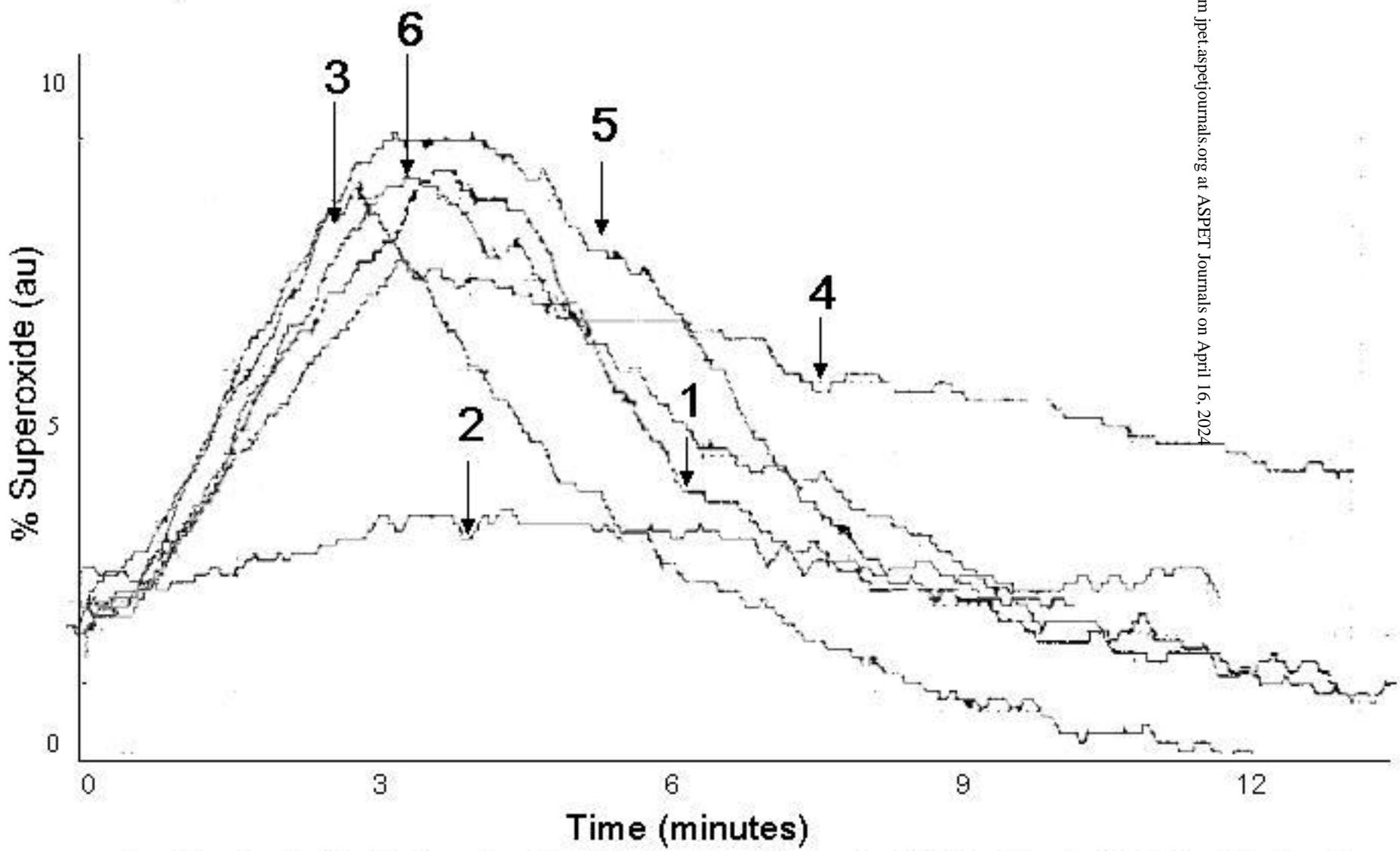


Figure 4



1 = Control; 2=17-β-estradiol; 3 =Tamoxifen; 4 =NDM; 5 = 4-OH; 6= Endoxifen

Figure 5a

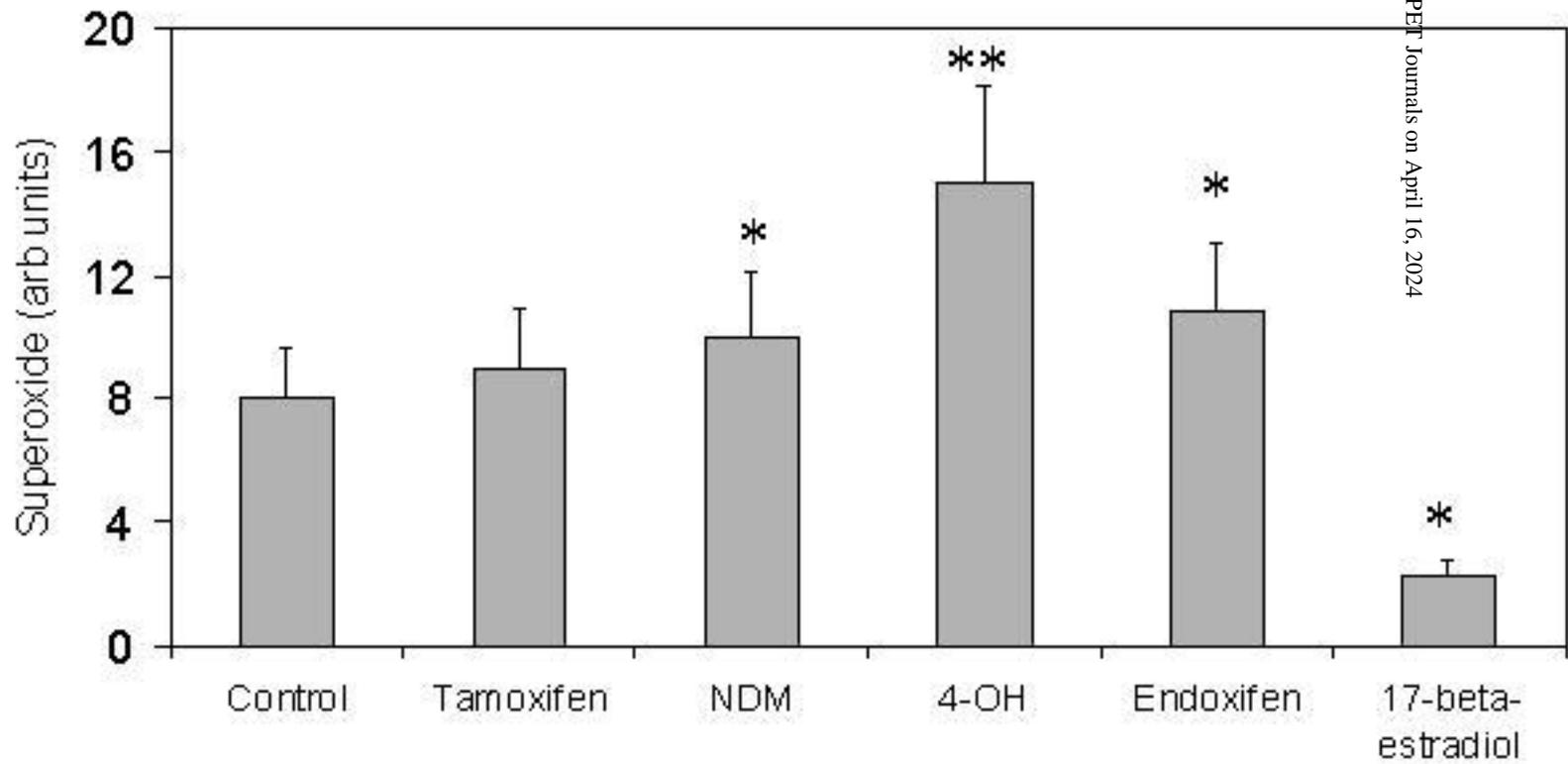


Figure 5b

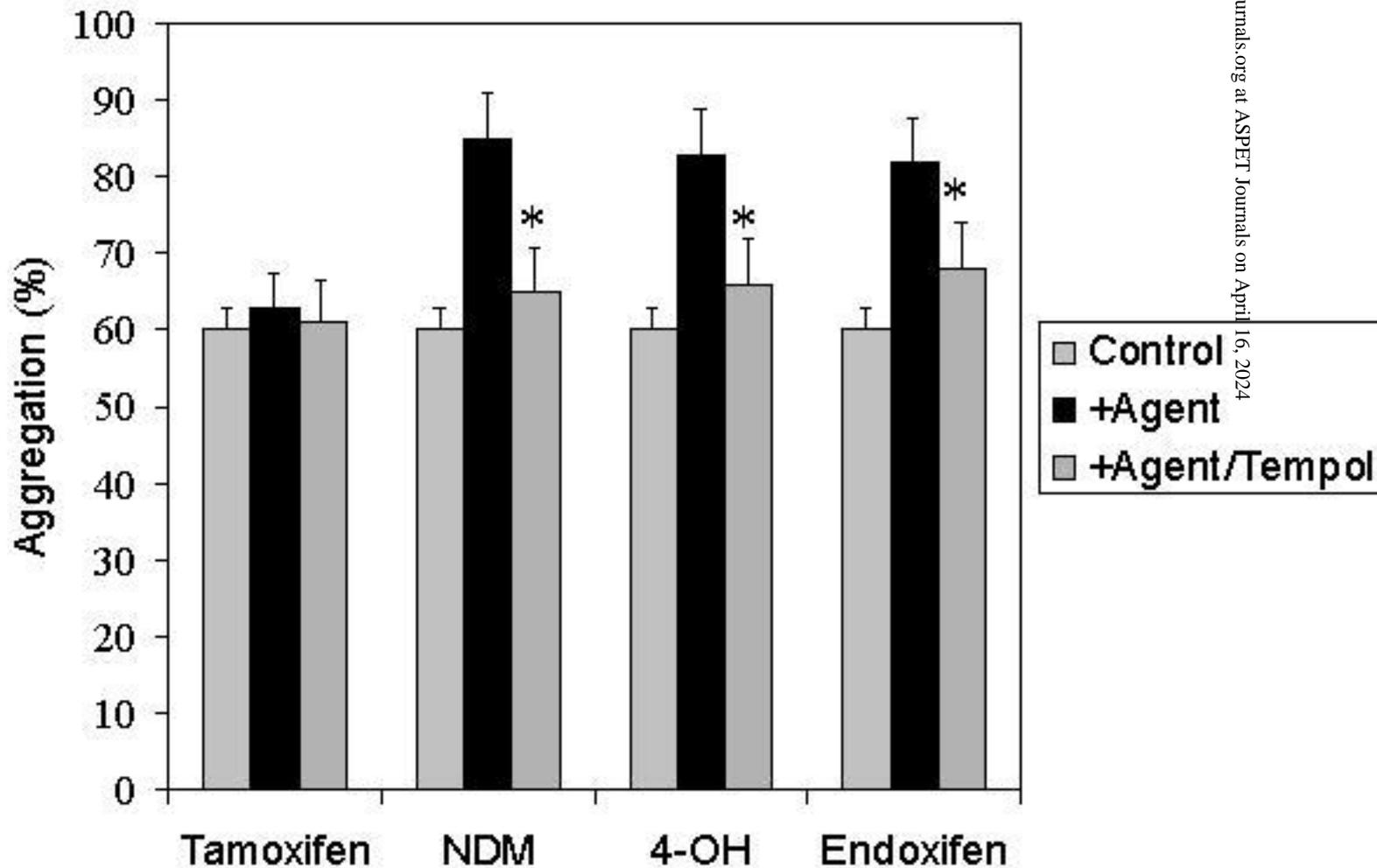


Figure 6

