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β-estradiol effect on erythrocyte aggregation – A controlled *in vitro* study [1]

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Abstract. The purpose of this *in vitro* study was to assess the effect of 17β-estradiol on hemorheologic parameters, namely on erythrocyte aggregation and deformability and membrane fluidity.

Blood samples from 65 women (aged 57 ± 4 years) undergoing postmenopausal hormone replacement therapy were obtained and were incubated for 5 min in absence and presence of 17β-estradiol 10^{-5} M. The measured parameters were the erythrocyte aggregation (EAI) and deformability (EI), the acetylcholinesterase activity (AChE), the plasma pH and osmolality and the erythrocyte membrane fluidity assessed by fluorescence polarization with two probes 1,6-diphenyl-1,3,5-hexatriene (DPH) and (1-(4-(trimethylamino)-phenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH). Data analysis was performed using t-Student and Pearson correlation analysis.

A statistically significant decrease of the EAI (15.8 ± 3.02 vs 13.45 ± 2.3; $p < 0.001$) and an increase of the EI (51.39 ± 5.64 vs 52.06 ± 5.36; $p < 0.01$) at shear stress of 30 Pa in presence of 17β-estradiol 10^{-5} M was obtained. There was a decrease in membrane fluidity in 45 blood samples (DPH) and in the other 20 an increase, when β-estradiol 10^{-5} M was present.

In vitro β-estradiol 10^{-5} M decreased erythrocyte aggregation in blood samples of postmenopausal women undergoing hormone therapy, which could prevent high blood viscosity and, consequently, cardiovascular events.

Keywords: β-estradiol, erythrocyte aggregation, membrane fluidity

1. Introduction

17β-estradiol as free radical scavengers is a steroid hormone, unionised and lipophilic at physiological pH. Fifteen to 35% of the total amount of steroids in the blood is transported by red blood cells that carry 17β-estradiol, both membrane-bound (two thirds) and in the cytoplasma (one third of the 17β-estradiol) being the erythrocytes responsible for 5–15% of sex hormone delivery to target tissues [1,2]. Its importance has been lately pointed out for its cardiovascular risk reduction in postmenopausal women [3] although the mechanism of this cardioprotective effect remain unclear. An antioxidant role as free radical scavengers has been demonstrated which could explain the protective role [4,5]. The work of Mc Manus et al. [6] did not support the role of oestrogens as antioxidants *in vivo*, besides the lower plasma hydroperoxide level determined four weeks after insertion of the estradiol implant. Previously data in primate have been shown that chronic 17β-estradiol supplementation decreased LDL accumulation *in vivo* [7].

Several studies on estrogen effects on the blood vessel wall are arising [8,9] and it was suggested previously an increasing production of nitric oxide induced by estrogen, that may contribute to the improvement in myocardial metabolic disfunction by increasing coronary blood flow [10]. An increase in

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blood flow in the peripheral arteries was verified after administration of hormone replacement therapy in women who had undergone surgical menopause [11].

So in what concerns the hemorreologic properties Coata et al. showed that low-dose triphasic contraceptives does not seem to affect blood viscosity [12] and in another study it was verified that the estrogen replacement therapy lowering plasma viscosity in a group of 23 women [13].

The effect of 17β-estradiol on membranes has been the aim of some studies focusing epithelial cell membranes that seemed to become more fluid [14]. Other studies, on microvilli, indicate that estradiol acts on the lipid composition of membranes, probably changing the phospholipids availability [15]. Moreover the membrane action of sex steroids on other elements of the erythroid line (reticulocytes) has been reported to be of different extent than the traditional nuclear action of steroids [16].

Jacobson et al. mentioned that steroid binding to membrane proteins is associated with short term cytophysiological events such as ion fluxes. However, they believe long-term genomic responses involving intracellular receptors follows another route determined by membrane lipid [17], which shows the relevance of the membrane properties and subsequently the hemorheological importance of estradiol in blood. Recently Levin's group also showed that estrogen, via a signal transduction pathway governed by membrane receptors, could both rescue endothelial cells from a hypoxia induced death and promote new blood vessel formation [18].

As mentioned above the β-estradiol is transported in blood by erythrocyte in a membrane bound way so, it seems possible the occurrence of some erythrocyte functions and properties modifications in presence of estradiol.

Therefore the aim of this study was to determine the 17β-estradiol effect on the hemorheological and biochemical properties of postmenopausal women erythrocytes.

2. Material and methods

2.1. Subjects

Subjects in this study were out-clinic patients in the Gynecology Department of the Santa Maria Hospital, Lisbon, Portugal, where they had been subjected to total hysterectomy for fibromyomas. Sixty-five Caucasian women using transdermic 17β-estradiol (50 μg/dia), as hormonal replacement therapy (HRT), were volunteer for this study. No subjects used any other medication besides the above mentioned HRT. They were screened for diabetes, renal failure, hypertension, cardiac disease and thrombosis and were considered to be apparently healthy. The 65 women aged 57 ± 4 years old.

2.2. Blood sampling and incubation procedure

Venous blood (5 ml) was obtained after 15 min in the recumbent position with previous consent and collected in tube with anticoagulant (10 I.U. of heparin/ml) and total blood was divided in two aliquots and centrifuged for 1 min, at 12 000 rpm (5414 Centrifuge Eppendorf, Sotel).

Ten microliters of plasma were replaced by the same amount of either physiological serum or a 17β-estradiol (water soluble from Sigma E-4389) solution, so that the final concentrations of 17β-estradiol in blood aliquots would be 0 M (control) and 10^{-5} M in the range of physiological concentrations. The two aliquots were incubated for 5 min, at room temperature, on a shaking table IKA-vibrax-UXR.

2.3. Measurements

Erythrocyte aggregation was assessed using the aggregometer Myrenne MA1 from Myrenne (Roentgen Germany) with 20 μl of blood. The final value considered is the average of three measurements, performed in stasis during 10 seconds after difusion of the blood sample.

The erythrocyte deformability for different shear stress (0.30; 0.60; 1.20; 3.00; 6.00; 12.00; 30.00 and 60.00 Pa) was determined using the Rheodyn SSD laser diffractometer from Myrenne (Roentgen, Germany) and the erythrocyte deformability is expressed as the elongation index (EI) in percentage. The erythrocyte acetylcholinesterase (AChE) activity was assessed by the Kaplan's colorimetric method [19], and the results expressed as units/minute/mg hemoglobin and the hemoglobin concentration was determined in an Hemoxymeter OSM3 Radiometer.

A ABL 505 electrode system from Radiometer was used to measure plasma pH.

For assessing plasma osmolality, the blood aliquots were centrifuged again under the same conditions, 75 μl of plasma were removed and used in the osmometer Osmomat 030.

The erythrocyte membrane fluidity was assessed determining fluorescence polarization using two fluorescent probes: for the membrane external layer, TMA-DPH 1-(4-(trimethylamino-phenyl-1,6-phenylhexa-1,3,5-triene) and for the hydrophobic portion, DPH (1,6-diphenyl-1,3,5-hexatriene). The fluorescence polarization intensities were recorded at excitation wavelength (λ_{exc}) 340 nm and emission wavelength (λ_{em}) 425 nm when TMA-DPH was used and λ_{exc} of 352 nm and λ_{em} of 430 when DPH was assessed [20,21].

Intracellular calcium (Ca^{2+}), was measured using the fluorescent dye fura-2 acetoxymethyl ester (Fura 2-AM) as described by David-Dufilho [22]. Briefly erythrocyte (10^8 cells/ml) were loaded with 0.5 μM Fura 2-AM and incubated during 45 minutes and then diluted at 10^7 cells/ml for measurements. Fluorescence intensities were measured at λ_{exc} 335 nm and λ_{exc} 385 nm with fixed emission wavelength of 515 nm and its ratio is a indicative of intracellular calcium concentration, and the results were expressed in nM.

All the fluorescence spectroscopy measurements were carried out in a Hitachi F-3000 fluorescence spectrophotometer (Tokyo, Japan).

2.4. Statistical analysis

Results are expressed in average $\pm$ standard deviation. As for software "SPSS for windows" was used. Bilateral t-Student test for paired samples was applied, being the null hypothesis rejected for a significance level of $p = 0.05$. Pearson correlation among all the variables considered was done.

3. Results

The summary of the results obtained with [17β-estradiol] $= 10^{-5}$ M vs [17β-estradiol] $= 0$ M is shown in the Table 1.

A statistically significant decrease in the erythrocyte aggregation was obtained for [17β-estradiol] $= 10^{-5}$ M ($p < 0.001$), relatively to the control aliquot. On the other hand, the erythrocyte deformability (shear stress 30 Pa) was significantly increased in the presence of [17β-estradiol] $= 10^{-5}$ M ($p < 0.01$), when compared to the control aliquot. In what concerns plasma osmolality, an increase was verified. In the presence of [17β-estradiol] $= 10^{-5}$ M for a $p < 0.01$, when compared to the control.

Table 1

Values (mean $\pm$ standard derivation), of the erythrocyte aggregation (EAI), the elongation index (EI) at shear stress of 30 Pa, plasma osmolality, pH, intracellular erythrocyte calcium, erythrocyte acetylcholinesterase (AChE), fluorescence polarization (determined with DPH and TMA-DPH), obtained in blood sample aliquots in absence (control) and presence of 17β-estradiol 10^{-5} M

	Control aliquot	17β-estradiol 10^{-5} M	
EAI (nd)	15.2 ± 3.0	13.4 ± 2.3	$p < 0.001$
EI (%) 30 Pa	51.39 ± 5.64	52.06 ± 5.32	$p < 0.01$
Plasma osmolality, mOsm/kgH_2O	0.285 ± 0.006	0.424 ± 0.028	$p < 0.001$
pH	7.48 ± 0.05	7.49 ± 0.067	$p < 0.02$
Ca^{2+}, nM	72.51 ± 19.27	80.46 ± 23.03	$p < 0.001$
AChE, units/min/mgHb	298.6 ± 30.4	294.4 ± 27.0	n.s.
DPH (nd)	0.289 ± 0.031	0.296 ± 0.029	n.s.
TMA-DPH (nd)	0.318 ± 0.011	0.322 ± 0.015	n.s

For the plasma pH a statistically significant increase was also observed in the aliquot with [17β-estradiol] $= 10^{-5}$ M ($p < 0.02$), relatively to the one without estradiol.

The results obtained for the erythrocyte acetylcholinesterase enzyme activity, for fluorescence polarization assessed with TMA-DPH and DPH fluorescence probes were not statistically significant, for the chosen significance level.

However, for the DPH values, another analysis approach was used in which the 65 samples was subdivided in two groups (one with 20, the other with 45) and there seem to appear curious results. These two "sub-populations" seem to behave differently. For the 45 samples sub-group, there was a statistically significant increase in the DPH values (decreased membrane fluidity), for [17β-estradiol] $= 10^{-5}$ M ($p < 0.01$), relatively to the control. On the other hand, in the other 20 samples a statistically significant decrease (increased membrane fluidity) for the same probe was found for [17β-estradiol] $= 10^{-5}$ M when compared to [17β-estradiol] $= 0$ M (Fig. 1).

The Pearson correlation analysed for all the parameters, doing all the possible crossings, was not significant.

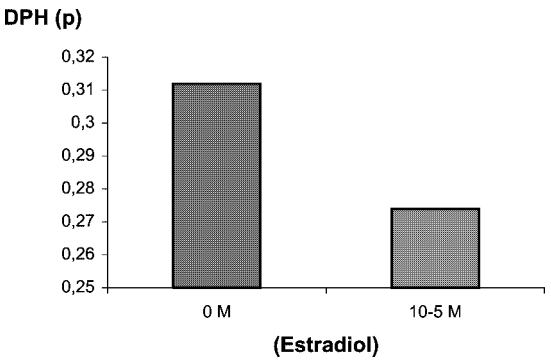

Fig. 1. Mean values of fluorescence polarization obtained with the probe DPH in 20 blood samples aliquots in absence (0 M; 0.311 ± 0.026) and presence of 17β-estradiol 10^{-5} M (0.273 ± 0.021) which were significantly decreased ($p < 0.01$.)

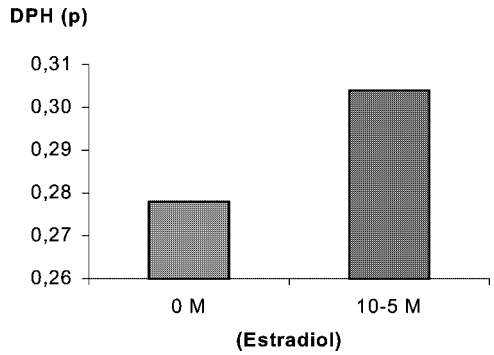

Fig. 2. Mean values of fluorescence polarization obtained with the probe DPH in 45 blood samples aliquots in absence (0 M; 0.278 ± 0.27) and presence of 17β-estradiol 10^{-5} M (0.306 ± 0.029) which were significantly increased ($p < 0.01$).

4. Discussion

In this study, the effect of 17β-estradiol 10^{-5} M on several hemorheologic parameters was evaluated. It was determined a decrease in the erythrocyte aggregation as well as an increase the in erythrocyte deformability for a high shear stress (30 mPa). These results suggest the ability of estradiol to favour the mobility of these cells, making them keener to adapt to the differents vessels, mainly may affecting favourably the microcirculation physiology. It obviously may prevents a high blood viscosity, and consequently normal blood flow behaviour which may be a contribution to the cardioprotective role of the hormone replacement therapy as it was verified. The mechanism for the explanation of the β-estradiol effects on erythrocyte functions was not yet our focus, but other authors mentioned estradiol eventual effects on cells cytoskeleton. A study performed with cell cultures demonstrated that estradiol was capable of preventing the disruption of F-actin which integrity is necessary for creating a selective vascular permeability barrier to proteins by endothelium cells [18]. The presence of progesterone in the incubation medium containing estradiol did not modified the red blood cell deformability of both groups of women, the healthy one and the other with sickle cell disease [23]. The results obtained in an *in vitro* study with platelets incubated with β-estradiol indicated an inhibitor effect of estradiol in platelet aggregation stimulated previously with thrombin or ADP [24]. In our work the decrease of erythrocyte aggregation verified in presence of β-estradiol, besides the increase values of plasma osmolality, suggest another kind of mechanism in antagonism to the strong tendency of erythrocyte to undergo aggregation in media of higher osmolality [25].

In fact, estrogens are known to interact *in vitro* with ion channels and ATPases from rabbit reticulocytes [16], and these interactions can also probably explain the increased values observed in our work both in the plasma osmolality and pH if the multiple ionic changes that could be involved were considered.

Estradiol is proved, for instance, to close K^+-ATP channels [26]. However some more studies could be interesting to explore which elements are exactly the most relevant in this process. From our study, it can only be concluded that erythrocyte membrane integrity was not affected because the AChE activity recognised as a maker was not changed by estradiol (10^{-5} M) [27].

In what concerns the membrane fluidity, there was only significant changes for the hydrophobic layer (DPH), when two sub-populations of blood samples aliquots were considered ($n = 45$ and $n = 20$). The opposite membrane fluidity results (decrease vs increase) obtained for the two sub-populations suggests that there are two different types of membrane "behaviouring" with reaction to estradiol, which could be related to the multiple possible interactions of the estradiol molecule with the different kinds of lipids

of the membrane. A similar dual behaviour was described for the cholesterol effects on the membrane fluidity which it may act as a fluidizer or at variance increasing the rigidity [28]. Moreover considering the antioxidant effect [29] and molecular similarities of estradiol and some lipids, it can probably be imagined that estradiol can reach the hydrophobic layer and from there eventually interfere with other membrane molecules or even transduce a message to the interior of the cell. The mechanisms for this action is still unknown, but Golden et al. [30] have been determined the localization of 17β-estradiol at 0–7 Å from the red cell membrane bilayer center deep within the hydrocarbon core. The estradiol role on membrane fluidity of red blood cells from women submitted to estradiol treatment for 5 week period was different from our *in vitro* study and an increase in erythrocyte fluorescence anisotropy was obtained [31]. In our work in presence of estradiol 10^{-5} M the intracellular calcium concentration increased significantly (Table 1) and previous studies suggested that estrogens affected intracellular calcium levels possibly by the phospholipase C cascade [18,32] or by hormone regulated Ca^{2+}-ATPase activity [16]. Picotto et al. [32] have been reported a non-genomic modulation of intestinal cell Ca^{2+} homeostasis by 17β-estradiol, and his work shows that the phospholipase C transmembrane signal is also involved in the non-genomic action of estrogen in intestinal cells.

Changes in cellular calcium level may occur as a result of increased calcium influx, decreased calcium eflux on the combination of both process. The study of Gafter et al. [33] have been confirmed a gender related difference in the intracellular calcium concentration which is higher in women. It is suggested that the increased red blood cell calcium concentration in women and female rats is most probably associated with their estrogen. The authors have been suggested that the enchancement of calcium influx into RBC seems to be the estrogenic mode of action. The same authors verified that the Ca^{2+} ATPase activity was not affected by a large range of β-estradiol concentrations. In our work the increase intracellular calcium concentration was not correlated with membrane fluidity possibly because its nanomolar level was not enough to affected membrane fluidity as have been demonstrated in Watanable et al. study [34] using eryhthrocyte incubations with 2.8 mM $CaCl_2$.

Besides the increase in intracellular calcium concentration, the erythrocyte deformability increased at 30 Pa which could be a result of the calcium antagonistic properties of 17β-estradiol reported by others in *in vitro* study [35,36]. However if the increase calcium intracellular concentration verified in our study will occur with impaired sodium efflux (probably with water influx) this hypothesis could explain the increased plasma osmolality obtained.

5. Conclusion

As a conclusion, even though more studies are necessary, this work points out relevance of the steroid hormones effect, on membranes, mainly on such an important one as the erythrocyte membrane. It also reckons estradiol (10^{-5} M) effect/action as a facilitator of the mobility of the red blood cell, preventing high blood viscosity, which might come in handy for clinical application in vascular diseases.

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