Effect of coagulation time on the concentration of epitheliotrophic components of autologous serum.

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Research article

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Abstract

Background: To know the influence of coagulation time on the concentration of epitheliotropic factors of autologous serum.

Methods: A prospective, cross-sectional and experimental study was conducted evaluating the concentrations of epitheliotropic factors in the serum of 20 healthy volunteers over 18 years of age, who did not suffer from diseases or took any medication or with a history of blood transfusion in the last year, those with diagnosis of anemia, coagulation diseases or another situation that contraindicates the sampling were excluded. Epidermal growth factor (EGF), Transforming growth factor β1 (TGF-β1) and Fibronectin were quantified in the various undiluted serum samples by an enzyme-linked immunosorbent assay.

Results: 65% were male with an average age of 22.7 ± 5.47 years (range 19 to 45 years). Age was similar in both groups (p = 0.87), men had an average age of 22.85 ± 6.84 years and women 22.43 ± 1.13 years. Vitamin A and Fibronectin did not have a statistically significant difference of concentration at 2 and 24 hours, while the concentration of TGF-β1 had a statistically significant decrease as the clot formation time increased (average 0.22 range, 0.003,0.451), opposite, the EGF increased its concentration statistically significantly with the longer coagulation time (average -0.39 range, -0.60, -0.18).

Conclusions: a prolonged clotting time (24 hours) have a significant impact on the composition and epitheliotrophic factors of the serum, increasing the EGF concentrations, and lowering the TGF-β1.

Introduction

Dry eye disease (DED) is a multifactorial illness of the ocular surface characterized by loss of the tear film homeostasis, and it is accompanied by visual symptoms derived from tear film instability and hyperosmolarity, damage and inflammation of the ocular surface, and neurosensory abnormalities [1]. The disease is caused by two conditions that can coincide, an abnormal function of the meibomian gland or a decreased tear production [1, 2] .

In the United States, the prevalence of DED in people over 18 years is around 6.8% and increases according to age (18–34 years 2.7%; >75 years 18.6%) being older in women (8.8%) than in men. (4.5%) [3]. It has been reported that up to 86% of patients who attend to general ophthalmology clinics present symptoms of dry eye, [4] and in patients attending to a third-level hospital eye care unit up to 94% present ocular damage [5].

To date, the most commonly used treatment for DED continues to be topical lubricants [2]. However, several therapies have been investigated and used to control the effects of the disease. In 1984, Fox et al. reported the use of autologous serum to treat patients with keratoconjunctivitis sicca [6]. The use of human autologous serum has been suggested to be advantageous over other currently available medications since it has multiple components similar to human tears [7] that are diminished in patients

with dry eye [8]. Among the elements present in autologous serum are Epidermal growth factor (EGF), vitamin A and Transforming growth factor β (TGF-β) and Fibronectin [9], the same three factors have been described to be among the most critical components of human tears [10]. EGF promotes corneal epithelial cell migration and proliferation, accelerating the wound healing process while TGF-β1 and TGF-β2 inhibit epithelial proliferation in a dose-dependent manner [7, 11–13]. It believed that Fibronectin secretion promotes the migration of corneal epithelial cells [7, 14, 15] similar to has been observed with Hyaluronic acid that supports the migration of corneal epithelial cells by accelerating the repair of the corneal epithelium [14].

Autologous serum has been widely used in patients with dry eye disease [12, 16, 17] and other ocular surface pathologies [13, 18] with variable results evidenced by two systematic reviews [19, 20], which suggest a strong influence of variations in preparation techniques on the final concentration of epitheliotrophic factors [21, 22]. Liu et al. studied the effect of centrifugal power (500 and 3000 g forces), centrifugation time (5 vs. 15 minutes) and the coagulation time (15, 30, 60 and 120 minutes) in the realization of the autologous serum in the concentration of various epitheliotrophic factors, as well as an in vitro proliferation, differentiation and migration model of corneal epithelial cells, using different centrifugal force and time resulted in significantly different concentrations of TGF-β1. EGF and vitamin A, without having a statistically significant effect on Human Platelet Derived Growth Factor (PDGF-AB), Fibronectin, and vitamin E. Concerning coagulation time, longer coagulation times produced higher concentrations of all epitheliotrophic factors. However, it was only statistically significant for EGF, TGF-β1, and Hepatocyte Growth Factor (HGF); additionally, found that with the same form of preparation (3000 g forces per 120 minutes), there was a higher concentration of TGF-β1 and fibronectin in young donors [21].

Since authors did not find studies that evaluate longer coagulation times, a study was conducted to assess differences among concentrations of EGF, TGF-β1, fibronectin, and vitamin A when human serum was exposed to an increased coagulation time of 24 hours.

Material And Methods

A prospective, cross-sectional, comparative, and experimental study was conducted to evaluate the concentrations of epitheliotrophic factors in the serum of 20 healthy volunteers. Participants included in this study were individuals older than 18 years without known pathological conditions, under any medical treatment, without a history of blood transfusion in the previous 12 months.

The study was approved by the Research Committee and the Bioethics Committee of the Health Sciences Division of the University of Monterrey. All participants signed informed consent before obtaining blood samples. The study adhered to the guidelines of the Declaration of Helsinki.

Autologous Serum Preparation

Two venous blood samples were obtained in 6 ml tubes (BD Vacutainer,_New Jersey, USA) without anticoagulant by ulnar venipuncture. To prepare the autologous serum, the protocol published by Liu et al. [21] was reproduced, using a centrifugal force of 3000 g forces with a time of 15 minutes (Thermo scientific Brand Refrigerated Centrifuge Model Sorvall, Thermo Fisher Scientific, Inc., Waltham, Massachusetts, USA), for comparison of coagulation times, blood samples from each participant were divided into two groups. Group A samples were kept at room temperature (18 to 25 ° C) for 2 hours, and Group B samples were held in the same conditions for 24 hours. After centrifugation, all the supernatant serum was carefully recovered in sterile conditions, divided into aliquots, and stored at -70 ° C serum for its further analysis.

Quantification of Epitheliotrophic Factors

Epidermal growth factor (EGF), Transforming growth factor β1 (TGF-β1), and Fibronectin were quantified in the various undiluted serum samples by enzyme-linked immunosorbent assay (ELISA) (R&D Systems; Minneapolis, MN, USA) and vitamin A concentrations were determined using spectrophotometry (Colorimetric, Novus Biologicals, Centennial, Colorado, USA) according to the manufacturer's instructions. All studies were analyzed on a spectrophotometer (Thermo scientific Model, Severalkan LUX Brand. Thermo Fisher Scientific, Inc., Waltham, Massachusetts, USA).

Sample calculation

To calculate the sample size due to the lack of prior information on the variable under study, similar studies were analyzed and convenience sampling was performed.

Statistical analysis

The statistical analysis was carried out with the SPSS statistical package (SPSS 24.0 for Mac, SPSS Inc., Chicago, IL). Data were analyzed for normal distribution with Shapiro–Wilk test. All data were presented as mean ± SD. Differences in the

concentration of epitheliotrophic factors between 2 hrs and 24 hrs were compared by two-sided paired sample *t*-test or the non-parametric Wilcoxon test.

Results

Twenty samples from 20 healthy volunteers were studied, 65% of the participants were male with an average age of 22.7 ± 5.47 years (range 19 to 45 years). Age was similar in both groups (p = 0.87), men had an average age of 22.85 ± 6.84 years and women 22.43 ± 1.13 years.

Serum concentrations of vitamin A and fibronectin were not significantly different between the 2-hour and 24-hour samples (p = 0.15 and 0.95 respectively) (Table 1). Serum concentrations of TGF β1 were significantly lower in the 24-hour sample than in the 2-hour sample (p = 0.03, average 0.22 range, 0.003, 0.451), the EGF concentrations increased in a time-dependent manner (p < 0.001; average − 0.39 range, -0.60, -0.18). There were no significant differences in epitheliotrophic factors concentrations between

blood samples of men and women (Fibronectin p = 0.25, vitamin A p = 0.55, TGF β1 p = 0.93, and EGF p = 0.19) (Figs. 1 and 2).

Table 1
Mean concentrations of epitheliotrophic factors after 2 and 24 hours of coagulation.

Epitheliotrophic factors	**2 hours** *mean ± SD (range)*	**24 horas** *mean ± SD (range)*	**Difference** *(CI 95%)*	**P value**
TGF-B1 *pg/mL*	1.50 ± 0.42 (0.92,2.46)	1.28 ± 0.27 (0.93,2.12)	0.22 (0.003,0.451)	0.03
EGF *pg/mL*	0.45 ± 0.27 (0.03,0.86)	0.85 ± 0.24 (0.11,1.41)	-0.39 (-0.60,-0.18)	0.0003
Vitamin A *nmol/ml*	0.87 ± 0.18 (0.55,1.14)	0.79 ± 0.18 (0.52,0.67)	0.08 (-0.03, 0.19)	0.15
Fibronectin *pg/mL*	0.48 ± 0.57 (0.18, 2.88)	0.40 ± 0.20 (0.15, 1.02)	0.08 (-0.19,0.35)	0.95

Discussion

The healing mechanisms of the corneal epithelium require adequate cell proliferation, migration, intercellular and basement membrane adhesion [11], all these processes are mainly regulated by the epitheliotrophic factors, fibronectin, and vitamin A. The concentration of epitheliotrophic factors plays a preponderant role in the therapeutic effects of topical autologous serum [21], which are dose-dependent [23] and strongly influenced by the production technique [21, 22]. The results of this study show that concentrations of epitheliotrophic factors are modified by increasing coagulation times. This effect has been related to the role of platelet alpha granules as primary storage source of these epitheliotrophic factors [24], a longer coagulation time results in platelet disintegration as well as secondary aggregation [25, 26] and increased output of epitheliotrophic factors.

The present study shows that EGF concentrations increase in a directly proportional manner to coagulation time,. In contrast, TGF-β1 exhibits the opposite behavior, which is partially consistent with the results reported by Liu et al., who found an increase in EGF, TGF-β1 and HGF [21]. The reason why TGF-β1 decreases are not clearly understood.

The therapeutic effect of EGF is dose-dependent and mediated by a Fibronectin-dependent system [27]; its major effect is in the epithelium and corneal endothelium, where there are high and low-affinity receptors. At the same time, in keratocytes, there are only low-affinity receptors [11].

The TGF-B is a polypeptide that has pro and anti-scar functions, as it stimulates skin healing [28]. However, is also a potent inhibitor of epithelial and endothelial cell proliferation [7, 11–13], probably through the modulation of other epitheliotrophic factors such as EGF [23, 29].

Fibronectin is an indispensable component for the healing of the corneal epithelium promoting the proliferation, differentiation, and migration of the corneal epithelium as well as the production of collagen [15] [15], Like Liu et al. [21], in our study we did not find a statistically different fibronectin concentrations associated with increased clotting time, this is because plasma fibronectin is produced in the liver [30], so its concentration is not influenced by the time of clot formation [31].

Vitamin A is vital to keep the ocular surface healthy and helps repair processes [32], the lack of vitamin A causes xerophthalmia characterized by an abnormal differentiation of the ocular surface that results in corneal and conjunctival keratinization, ulceration, metaplasia squamous epithelial and loss of conjunctival goblet cells [33, 34] as well as increased synthesis of fibronectin [35]; The origin of this vitamin is in the diet [36], is stored in the liver and is transported in the blood by its specific receptor (retinol-binding protein) [37]. In our study we did not find a statistically significant change in vitamin A concentrations, this agrees with what was reported by Mejia and collaborators who studied the changes in vitamin A concentration by modifying the separation time of serum and clot 2, 4, 6, 12 and 24 hours, finding that at room temperature (26 to 28 º C) the concentration was stable for up to 24 hours [38] this could be since the retinol-binding protein does not change with the formation time of the clot.

EGF, and Fibronectin promotes corneal epithelial cell migration and proliferation, and TGF-β1 inhibit epithelial proliferation in a dose-dependent manner so longer coagulation time increases EGF and decreases TGF-β1 could improve corneal epithelial cell migration and proliferation.

This study has some limitations; the first one is the sample size; however, our sample size is similar to that of other studies [10, 16, 17, 21, 39–42]; since, due to the cost of supplies, it is difficult to include more patients. In addition, our study population it´s mainly of young adults, and according to Liu et al. age of the donors has an impact on Fibronectin and TGF-β1 concentrations [21], we cannot ensure that the effect of coagulation time is similar in all age groups. Finally, the clinical impact or therapeutic effects of the changes in the concentrations found was not evaluated.

In conclusions, a prolonged clotting time (24 hours) have a significant impact on the composition and epitheliotrophic factors of the serum, increasing the EGF concentrations, and lowering the TGF-β1.

List Of Abbreviations

TGF-β1= Transforming growth factor β1

EGF = Epidermal growth factor

DED= Dry eye disease

UDEM= University of Monterrey

PDGF-AB= Human Platelet Derived Growth Factor

HGF= Hepatocyte Growth Factor

95% CI= 95% confidence interval

Declarations

Ethics approval and consent to participate

Approval of the authorities and the Ethics Committee of the Universidad de Monterrey (UDEM) and adhered to the principles of the Declaration of Helsinki. After explaining the study, informed consent was obtained from all participants.

Consent to publish

Not applicable

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request..

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The authors declare that sponsors didn’t have any involvement in the design and realization of this paper.

Authors’ Contributions

AMC contributed to this paper by designing the work, designing the collection form, recollecting data by interviewing the subjects, writing the paper, and editing the work in order to submit it.

KGG contributed to this paper by designing the work, designing the collection form, recollecting data by interviewing the subjects, writing the paper, and editing the work in order to submit it.

MAG contributed to this paper by designing the work, designing the collection form, analyzing and interpretation of all data, writing the paper, and editing the work in order to submit it.

RBRLC contributed to this paper by designing the work, designing the collection form, analyzing and interpretation of all data, writing the paper, and editing the work in order to submit it.

MGL contributed by designing the work, designing the collection form, the conception of the paper, analysis and interpretation of the data, creation of the graphical information writing the paper, interpreting data, and editing the work in order to submit it.

All authors have read and approved the manuscript.

Competing interests

Manuel Garza-Leon, is on the speaker's bureau for Alcon. Allergan income from lectures. SIFI income from lectures.

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Figures

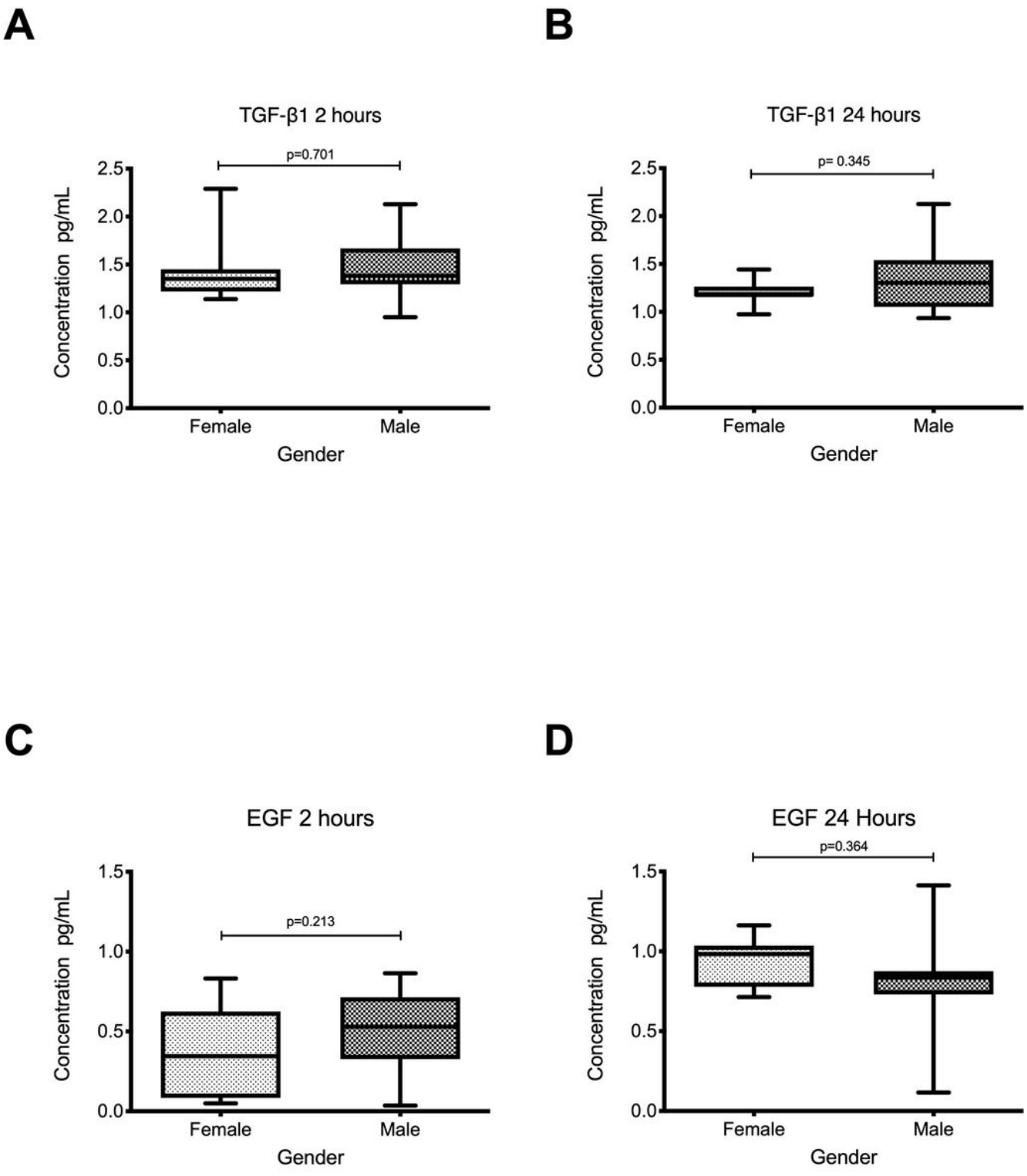

Figure 1

Gender differences between 2 (1A and 1C) and 24 (1B and 1D) hours coagulation time in Transforming growth factor β (TGF-β) and epidermal growth factor (EGF).

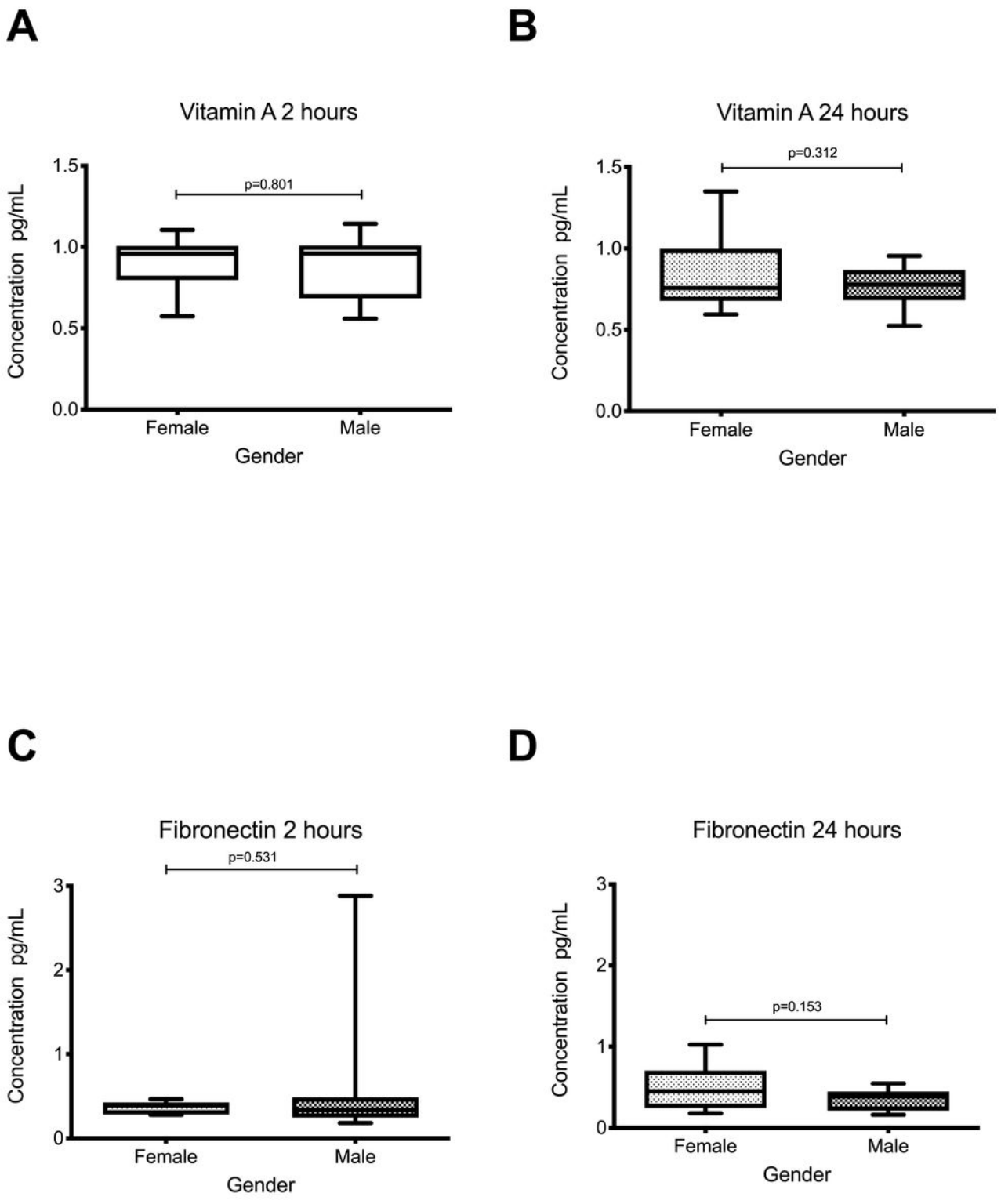

Figure 2

Gender differences between 2 (2A and 2C) and 24 (2B and 2D) hours coagulation time in vitamin A and Fibronectin.