ORIGINAL ARTICLE

ENDOSTATIN CONCENTRATION IN PLASMA OF HEALTHY HUMAN VOLUNTEERS

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Background: Angiogenesis is involved in many cardiovascular and cancerous diseases, including atherosclerosis and is controlled by a fine balance between angiogenic and angiostatic mediators. Endostatin is one of the main angiostatic mediators, and inhibits angiogenesis and prevents progression of atherosclerosis. The available literature shows a broad range of concentrations in relatively small samples of healthy controls and is calculated by using different techniques. This study was aimed to determine the basal endostatin concentration in plasma of healthy volunteers, to fully understand its physiological role. **Methods:** Fifty healthy adult volunteers were recruited to the study. Participants were advised not to participate in any physical activity on the day before the blood sampling. The volunteers' physical activity, height, weight, heart rate and blood pressure were recorded. The samples were analysed for plasma endostatin concentration, using ELISA. The participants were divided by gender and ethnic groups to calculate any difference. **Results:** Endostatin and other variables were normally distributed. Most of the participants had a moderate level of physical activity with no gender related difference (p=0.370). The mean value for plasma endostatin in all samples was 105±12 ng/ml with range of 81–132 ng/ml. For males, it was 107±13 ng/ml, while for females; 102±12 ng/ml. There were no significant gender or ethnicity related differences in endostatin concentration. Moreover, endostatin was not significantly related with any anthropometric and physical variable. **Conclusion:** This study gives endostatin levels in normal healthy people and show no gender and ethnicity related differences in endostatin levels. Endostatin was not related with any anthropometric and physical variable.

Keywords: Endostatin; Vascular endothelial growth factor; Angiogenesis; Atherosclerosis; Ethnic groups

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INTRODUCTION

Angiogenesis contributes to the progression and maturity of many cardiovascular and cancerous diseases including atherosclerosis.[1] There is a fine balance between the angiogenic and angiostatic mediators in the overall process of angiogenesis and shift in favour of any particular phenotype determines the outcome of angiogenesis.[2] The angiogenic mediators like VEGF and FGF favour angiogenesis, while endogenous angiostatic mediators, including angiostatin and endostatin tend to stop the angiogenic process.[3] Endostatin, discovered by O'Reilly *et al* in 1997, has come to the front as one of the main angiostatic mediators.[4] Endostatin plays an essential role in control of the angiogenesis. By inhibiting angiogenesis, endostatin prevents progression of atherosclerosis[5], primary growth of tumours and metastasis.[4]

Endostatin is a small globular protein attached through a hinge to the C terminus of collagen XV and XVIII in the basement membrane of endothelial cells.[6] It is also found in many other body tissues including heart, skeletal muscles, liver, placenta, ovaries, prostate and kidneys.[7] The available literature contains several studies reporting the concentration of endostatin in the plasma (Table-1). Table-1 show that the sample populations are often small and drawn from patients with a range of pathologies. Moreover, shows a broad range of concentrations in relatively small samples of healthy controls. In addition, a variety of analytical techniques have been used. Thus the current literature does not provide the average plasma concentration in healthy human population.[8] Some studies have reported the plasma endostatin concentration as low as 1.0 ng/ml while others have reported this level to be in the range of 300 ng/ml (Table-1). These huge differences in means and ranges are not completely clear. It is clear from the table, that for the determination of endostatin concentration in plasma, different methods like mass spectrometry, radioimmunoassay, western blots and ELISA have been used frequently.

The difference in the reference range could possibly be due to use of different techniques. However, even the same techniques used in different studies have reported significant differences in the basal plasma endostatin concentrations.[9,10] This study was aimed to determine the basal endostatin concentration in plasma of healthy volunteers. In addition, difference in basal endostatin concentration with gender, age, height,

weight, and physical activity behaviour of the individuals will also be evaluated.

MATERIAL AND METHODS

The experimental protocol was approved by the University of Glasgow, College of Medical, Veterinary and Life Sciences Ethics committee. All recruitments and experimental procedures were carried out in the different labs of school of Life Sciences. Volunteers were enrolled to the study, after initial screening by asking questions about the inclusion and exclusion criteria. The participants belonged to 3 main geographical regions, Europe, Asia and Middle East.

Participants were advised not to participate in any physical activity on the day before the blood sample. In total, 50 healthy adult volunteers were recruited to the study. The characteristics of the volunteers are summarised in table-2, which subdivides the data by gender to allow comparison. Another table-3 shows these data by geographical origin of the volunteers.

After giving informed consent, the volunteers filled out the International Physical Activity Questionnaire (IPAQ short form). The weekly physical activities of the participants were determined from IPAQ questionnaires and were categorised in low, moderate and high activity groups. The activity in MET.min.week^{-1} was also calculated numerically, according to the data processing rules given in the guide book.[11] These are given in table-2. The height, weight, heart rate and blood pressure of the volunteers were recorded. After collecting blood, it was centrifuged within 30 minutes and plasma was stored at -80°C. All samples were thawed and brought to room temperature before analyses. The samples were analysed for plasma endostatin concentration, using Quantikin^{R} ELISA kit bought from R&D systems. The samples were run in duplicates after dilution following the instructions of the manufacturer. A series of standards and controls were provided with each kit and these were used to generate calibration plots.

Statistical analyses were carried out using SPSS 17. The normality of the data was tested using Kolmogorov- Smirinov and Shapiro-Wilk tests. Summary statistics were calculated. Independent sample *t*-tests were used to investigate difference between means for males and females. ANOVA was applied to check the difference between means of participants from different ethnic groups. Pearson correlations tests were used to investigate the correlations of endostatin with anthropometric and physical characteristics of the volunteers. Uni-variate and multivariate regressions analysis was done to investigate association of endostatin concentrations with other variables. Categorical data of physical activity was analysed using Chi-square tests. The data was analysed for different types of physical activity both in categorical (high, moderate and low physical activity) by Chi-square, and continuous forms (MET-min per week) by independent sample *t*-test. *p*-value less than 0.05 was accepted as significant.

RESULTS

Results were obtained for all 50 volunteers. All the data were normally distributed including MET minutes. Males were significantly older (p=0.021), taller (p<0.001) and heavier (p=0.006) than females (Table-2). No statistical differences were observed for other variables. Most of the participants had a moderate level of physical activity (Table-2). Chi square test showed no significant difference in physical activity levels between male and female volunteers (p=0.370). The mean value for plasma endostatin in all samples was 105±12 ng/ml (Mean±SD). The range of concentration observed in this data set was from 81 to 132 ng/ml.

The mean endostatin concentration for male was 107±13 ng/ml, while for females; it was 102±12 ng/ml. When mean concentrations for both groups were compared, no significant difference was observed (p=0.21). The mean endostatin concentration for Europeans was 105±14 ng/ml, and for Asians was 106±13 ng/ml, while for Middle Eastern was 106±7 ng/ml (Table-3). No significant difference in endostatin concentration was found between ethnic groups (p=0.80). The difference in their physical characteristics are summarised in table-3.

Correlation of endostatin with anthropometric and physical variables was checked in pooled data and also in data split by gender and ethnicity. In pooled data, there was no significant correlation. However, in male individuals, there was a significant negative correlation between heart rate and endostatin concentration (supplementary table 1). In data split by ethnicity and sex, no correlations were found for height, weight, SBP, DBP, BMI and MET minutes with endostatin concentration. Strong negative correlation was observed in Middle Eastern females for heart rate. South Asian males were positively correlated for age and DBP (supplementary table-2). In addition, the regression analyses were also inconclusive in providing any association between different anthropometric variables and plasma endostatin concentrations, as shown in table 4. In uni-variate analysis heart rate and physical activity were significantly related with endostatin, however, no variable was related with endostatin in multi-variate analyses (Table-4). The overall value for R^2 in multi-variate analyses was 0.182 (18%).

Table-1: Evidence table of previous studies with different endostatin concentrations

Reference	Mean [ES] Plasma	Mean [ES} Serum	Mean Age years	Assay Method	Comments
Standker *et al* 1997[12]	10^{-10}M			Mass spectrometry	Human plasma hemofiltrate was used for isolation of endostatin using mass spectrometry
Hebbar *et al* 2000[13]		Healthy volunteers = 9.9±9.7 ng/ml	48.5±12.7	Enzyme immune essay	50 patients with different levels of established sclerosis were evaluated for plasma endostatin concentration.
		Sclerosis patients 63.2±20.2 ng/ml	54.1±14.6		
Feldman *et al* 2000[14]		Controls =14.1 ng/ml (1.0–19.3 ng/ml) Renal cancer patient =24.6 (15.1–54 ng/ml)	Patients=48 Controls=age matched	Competitive enzyme immunoassay	Serum samples of at pre-nephrectomy stage of 15 renal cancer patients were analysed against fresh samples from 18 healthy controls
Zorick *et al* 2001[9]		Downs patients (38.6±20.1 ng/ml) Healthy controls (20.3±11.5 ng/ml)		ELISA	Endostatin concentrations of Down syndrome patients were almost double to that of normal healthy volunteers.
Feldman *et al* 2001a[15]	colorectal cancer patients (71.6±28.6 ng/ml)		60 years	Competitive enzyme immunoassay	High endostatin concentrations were found in plasma of 30 colorectal cancer patients. Elevated levels were associated with disease progression.
	Healthy controls (43.2±15.1 ng/ml)		56 years		
Feldman *et al* 2001b[16]		controls=25.8 ng/ml Sarcoma patients=43 ng/ml	Patients=44 yrs, Age matched for controls	Competitive enzyme immunoassay	Serum samples from 25 soft tissue cancer patients in pre-surgery states were analysed for endostatin and compared to controls.
Gu *et al* 2004[17]	20.3±3.2 ng/ml		volunteers = 33±13	ELISA	7 healthy volunteers performed high intensity exercise. Their baseline concentrations were checked before exercise.
Teh *et al* 2004[18]	controls (34.97±3.76 ng/ml Patients (30.62±4.54 ng/ml)			ELISA	Post-operative breast cancer patients had a higher endostatin concentrations in plasma compared to controls.
Suhr *et al* 2007[10]	87.3±5.7 ng/ml		27.8±5.4	ELISA Western Blot	In this study 12 male cyclists perform exercise with and without hypoxia and vibration, and their effect on angiogenesis.
Sponder *et al* 2011[19]	Smoker elderly (103.3±16 ng/ml)		47.12 (39–62)	ELISA	Impact of exercise was checked on the plasma endostatin concentration in 17 elderly smokers, 20 non-elderly smokers, 19 young healthy non-smokers and 14 type 2 diabetics. Endostatin concentration was measured before and after exercise.
	Non-smoker elderly (116.3±15 ng/ml)		51.55 (36–70)		
	Young Non-smoker (93.4±15 ng/ml)		23.16 (18–34)		
	Diabetic (108.5±17 ng/ml)		57.86 (42–70)		
Sponder *et al* 2013[20]	Female athletes (129±21 ng/ml)		26±5	ELISA	Impact of bicycle exercise in athletes and sedentary males and females was checked. Baseline endostatin concentration was observed to be high in athletes than sedentary volunteers. Exercise increased the endostatin concentration in both groups.
	Female controls (89±15 ng/ml)		23±4		
	Male athletes (148±28 ng/ml)		24±4		
	Male controls (93±15 ng/ml)		23±4		
Makey *et al* 2013[21]		111±5 ng/ml	24 ± 0.8	ELISA	Low intensity exercise was performed in females belonging to African American (n=35, mean age 26.1±1.21) and Caucasian (n=37 mean age 22.1±0.84) ethnicities. No effect of exercise on plasma endostatin concentration was observed.

A chronological arrangement of the different studies reporting the endostatin concentrations in healthy and diseased population is shown. Endostatin concentrations are outlined as mean±SD with ranges, where possible. Age of the participants and techniques used for analyses of endostatin are also given in separate columns.

Table-2: Characteristics of the participants

	Total	Female	Male	*p*-value
Sample size (n)	50	16	34	
Age (Years)	26.7±7.80	23.1±4.4	28.5±8.5	0.021
Height (m)	1.71±0.07	1.66±0.1	1.74±0.05	<0.001
Weight (kg)	73.6±10.4	67.8±8.01	76.3±10.4	0.006
BMI (kg/m^2)	24.89±2.77	24.65±2.33	25.0±2.98	0.676
Heart Rate (bpm)	75±5	77±6	73±5	0.090
SBP (mm Hg)	123±9	118±12	125±7	0.059
DBP (mm Hg)	74±8	73±8	74±8	0.378
MET.min.week^{-1}	2471±1627	2572±1536	2424±1689	0.768
PA (High)%	38	50	32.4	0.370
PA (Moderate)%	52	37.5	58.8	
PA (Low)%	10	12.5	8.8	

p-value for the difference between males and females by independent sample *t*-test. Lower panel of table shows p-vale for *Chi* Square test for physical activity (PA). BMI; Body Mass index, SBP; Systolic blood pressure DBP; Diastolic blood pressure (values are presented as mean±SD), MET are the metabolic equivalent minutes of basal metabolic rate. PA; physical activity

Table-3: Anthropometric and physical characteristics of volunteers from different regional groups

		Europeans	Asians	Middle Eastern	*p*- ANNOVA
Sample size (n)	M	14	15	5	
	F	11	-	5	
	Total	25	15	10	
Age (years)	M	25±10.5	32±6.06	27.2±6.02	0.081
	F	21.2±1.6	-	27.6±4.5	0.009
	Total	23.3±8.1	32±6.06	27.4±5.04	0.004
Height (m)	M	1.75±0.04	1.74±0.05	1.74±0.04	0.60
	F	1.67±0.1	-	1.62±0.1	0.24
	Total	1.72±0.1	1.73±0.05	1.68±0.08	0.08
Weight (kg)	M	76.3±11.6	76.2±10.8	76.9±2.9	0.99
	F	68.9±8.3	-	65.6±7.8	0.46
	Total	73±10.7	76±10.8	71.2±9	0.15
HR	M	74±6	74±5	71±3	0.54
	F	76±6	-	78±5	0.60
	Total	75±6	74±5	74±5	0.97
SBP	M	127±5	122±9	127±2	0.11
	F	120±12	-	116±11	0.54
	Total	124±10	122±9	121±9	0.16
DBP	M	77±9	72±7	78±5	0.18
	F	73±8	-	73±10	0.96
	Total	75±9	72±7	75±8	0.48
Met.min.week^{-1}	M	2803±1704	2065±1695	2441±1747	0.50
	F	2850±1031	-	1958±2346	0.30
	Total	2824±1420	2065±1695	2200±1967	0.48
Endostatin (ng/ml)	M	107±15	106±13	108±7	0.90
	F	101±14	-	103±7	0.70
	Total	105±14	106±13	106±7	0.80

Values are mean±SD. *p*-value for ANOVA

Table-4: Regression analysis for endostatin with anthropometric and physical variables

Endostatin	**Uni-variate**		**Multi-variate**	
	Beta (CI)	*p*	Beta (CI)	*p*
Age	0.13 (-18.1–46.7)	0.38	-0.05 (-50–39.0)	0.81
Height	0.03 (-46–.355.4)	0.86	-0.55 (-6920–499.4)	0.75
Weight	-0.02 (-0.4–0.3)	0.87	0.78 (-6–7.8)	0.78
Sex	0.18 (-2.8– -12.5)	0.21	0.10 (-9–14.1)	0.65
BMI	-0.06 (-1.6–1.1)	0.69	-0.65 (-24–17.7)	0.77
SBP	0.16 (-0.2–0.6)	0.28	0.17 (0–0.8)	0.38
DBP	0.17 (-0.2–0.7)	0.23	0.04 (-1–0.6)	0.81
HR	-0.27 (-1.3–0.009)	0.05	-0.29 (-1–0.1)	0.10
MET_ min.week^{-1}	-0.21(-.004– .005)	0.15	0.06 (-.004–0.005)	0.84
PA Level	-0.23 (-10.2–0.9)	0.10	-0.27 (-16–5.5)	0.32

Uni-variate and multi- variate regression analyses for all variables were carried out, as shown by Beta coefficient (Beta) with confidence intervals (CI) and significance (P). Multivariate analyses R^2=0.182.

DISCUSSION

In our healthy study population, the mean value for plasma endostatin in all samples was 105±12 ng/ml with range of 81–132 ng/ml. For males, it was 107±13 ng/ml, while for females; it was 102±12 ng/ml. The mean endostatin concentration for Europeans was 105±14 ng/ml, and for Asians was 106±13 ng/ml, while for Middle Eastern was 106±7 ng/ml. There was no significant gender or ethnicity related differences in endostatin concentration. Moreover, endostatin was not significantly related with any anthropometric and physical variable. These results are consistent with the results published by Sponder; mean concentration of 93.4±15.3 ng/ml[19], and Suhr; 87.3±4.5 ng/ml[10]. The studies carried out by Sponder included 13 male healthy volunteers while the latter study had a sample size of 88. Another study published recently, reported mean endostatin concentration of 111±5 ng/ml, in 72 healthy females.[21]

However, these results are significantly different from the mean plasma endostatin concentration of 20.3 ng/ml reported by Gu[17], 14.1 ng/ml by Feldman[14] and 43.2 ng/ml by Teh[18], for healthy volunteers of different ages in their studies. These differences in concentrations could be attributed to the different techniques (ELISA, Western blot, Mass spectrometry etc) used for analyses. It should be noted from the studies listed in table-1, that more recent studies have reported comparatively high endostatin concentrations, which is pointing towards more sophistication and maturity of techniques with time.

The sensitivity of the ELISA kits from different manufactures might be a factor. Different studies using the same ELISA kits (Accucyte, Cytimmune Sciences Inc., MD, USA) have reported a low endostatin concentration[9,17], which could possibly be the reason for reporting low endostatin concentration. Moreover, the media used for analyses (plasma, serum, haemofiltrate) may have different sensitivity and specificity for the same techniques. Nonetheless, these differences are not completely clear because concentrations reported by the same author in different studies using the same techniques and media, show significant difference for healthy volunteers.[14,15] One might speculate about the causes of these differences. Serum concentrations of endostatin have been reported to be affected by circadian rhythms[22], age[23,24] and physical activity[17,23]. It was not possible to address these factors in our study.

The results in our study seems more reliable due to the larger sample size, good quality control indicators and the results lying within the range provided by the kit supplier. It is worth noting, that even this larger sample does not find volunteers with endostatin concentrations as low as those reported elsewhere.[15,17] It is also unlikely that geographical variations could explain these differences as only minor differences were observed in European, Middle Eastern and South Asian populations in our study. The second aim of the study was to investigate the difference in concentration of endostatin between males and females. The mean plasma endostatin concentration appears higher in male than female volunteers. However, the difference is not statistically significant (p=0.21). These results are in agreement with the result from other studies, where the male shows more mean concentration than female.[9,16] Though these studies have not mentioned any direct comparison between the two groups, the difference looks non-significant. In addition, another study which compared the concentration on gender basis also reported a slightly higher but non-significant concentration in males.[25]

The hypothesis tested in this study was that females might have lower concentrations of plasma endostatin. The basis of this expectation lies in the lower concentration of many biological molecules such as haemoglobin in females.[26] Gu speculated that endostatin in circulation might be due to physiological collagen turnover.[17] Based on this supposition, it can be assumed that more muscle mass in males may cause higher physiological collagen turnover which consequently might increase circulatory endostatin.

Plasma endostatin concentrations in volunteers of different ages were also investigated. The volunteers had a broad range (19–60 years) of age. However, age of 96% of the participants were 40 years and below. The oldest participant in the study was 60 years old male and his plasma concentration was 109 ng/ml, which is very near to the mean concentration. No significance correlation of plasma endostatin with age was found when the data from all volunteers was investigated. The data from the South Asian volunteers showed a positive correlation with age (p=0.049). Strong conclusion from this correlation cannot be drawn because; firstly, the p-value is border line to significance, secondly sample size is small (n=15) and thirdly by including the male participants from Middle Eastern and European group, significance is lost (p=0.52), as shown in table supplementary table-2. However, significantly higher endostatin concentration in older participants than young adults have been reported previously.[19,23] With advancing age the chances for angiogenic dependent diseases increases. It is possible that the circulatory endostatin concentration rises as an adaptive protective mechanism.

It was important to know that variations in physical and anthropometric characterises might affect the endostatin concentration in plasma. But the plasma endostatin concentration seemed uncorrelated with most of physical and anthropometric parameters such as height, weight, BMI and blood pressure. The author is unaware of any previous published data reporting these observations.

Physical activity has been widely accepted to decrease the risk of cardiovascular related mortality and morbidity.[27] The physical activities were assessed using the short form of International Physical Activity Questionnaire. They were categorised in low, moderate and high activity groups. The activity in MET.min.week^{-1} was also calculated numerically, according to the data processing rules given in the guide book.[11] METs refer to multiples of resting metabolic rate during an activity. MET-minutes are the MET score of an activity during a specific time period. The analysis revealed no significant relationship between plasma endostatin concentration and reported physical activity (p=0.816).

Higher concentrations of plasma endostatin were reported by Sponder in male and female athletes than in matched controls.[20] This difference in results could be due to several factors. Perhaps habitual physical activity has different effects from shorter periods of intense athletic training. It was assumed before the study, that regular physical activity might have an effect on the basal endostatin concentration. As participants were mostly students of the University of Glasgow, their involvement in physical activities was expected to be more than population in general. To minimise this effect, all volunteers were asked to abstain from physical exercise for 24 hours before the trial. However, under or over reporting of physical activities, unclear definitions of different intensities of activities, and the presence of the option "I don't know" in the questionnaire, could be the possible reasons for the data of physical activity being unreliable. More sophisticated approaches such as using accelerometers and GPS devices, to monitor the physical activity behaviour of the participants prior to blood sampling, could have provided more accurate information. Due to large sample size, cost of the GPS devices was one of the barriers in implementations.

The strengths of the study include; a wide sample size and inclusion of both male and female participants from different ethnic groups with wide range of age and other parameters including height, weight, BMI and heart rate. There were some limitations to the study such as more males (n=34) than females (n=16). This was not intentional and volunteers were recruited randomly. The results imply no difference on basis of gender and the imbalance between the number male and female participants may not have a profound influence on the results. However, the strength given to the results in setting the trends with a more evenly gender distribution, could not be rejected completely.

Plasma samples were run in duplicate on ELISA kits to calculate mean values for each subject as compared to the classical triplicate pattern used in many studies. There are two reasons for this: firstly, the ELISA manufacturer instruction says the plasma samples to be run in duplicate and secondly time and financial restraints. Triplicate results might have produced more accurate mean result but the good quality control indicators (inter and intra assays CV% <10 & 5% respectively) show that handling technique of the plasma samples, controls and standards, including pipetting and different steps in the analyses, were carried out accurately. The differences in mean endostatin concentrations were small and triplicates would have minimal effects on the overall results.

The experiment was not planned to investigate the difference in concentration in different regional groups. The regional groups were included only, when the initial statistics revealed no difference between the male and female participants and their demographic relationship with plasma endostatin concentration. This is also the reasons of the uneven distribution of participants in different ethnic groups. The results could be more reliable, had there been more participants in each group and study planned on more ethnic bases in the initial stage.

This study gives the endostatin levels in normal healthy people and show no gender and ethnicity related differences in endostatin levels. It also shows that endostatin was not significantly related with any anthropometric and physical variable. Based on this study's results, further research in to the effects of exercise on plasma endostatin concentration could be carried out without any specific gender or anthropometric requirements.

AUTHORS' CONTRIBUTION

IS collected and analyzed human blood samples, organised the data and wrote the manuscript. IS and RHB conceptualized the study and RHB did the final editing and approval. MOM organized the data, provided assistance in statistical analysis and wrote the manuscript. MJK provided assistance in statistical analysis and helped in writing and editing. SHH provided help in writing and final editing of the manuscript.

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