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Dehydroepiandrosterone Sulphate as a Skeletal Maturity Indicator - Cross Sectional Study

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ABSTRACT

Objective: The aim is to associate dehydroepiandrosterone sulphate (DHEAS) levels to skeletal maturity by using the cervical vertebra stages and its use as skeletal maturity indicator.
Results: Mean serum DHEAS levels were significantly higher in the post pubertal stages than in the pre pubertal and early pubertal stages. Linear correlation showed that DHEAS levels had a significant positive correlation with cervical skeletal maturity from the pre pubertal to the post pubertal stages.
Conclusion: Serum DHEAS could be used as a reliable indicator of skeletal maturity eliminating the risk of radiation exposure.

Keywords: dehydroepiandrosterone sulphate, cervical vertebra, skeletal maturity indicators, pre pubertal growth, post pubertal growth.

BACKGROUND

The developmental status of a child is usually assessed in relation to events that take place during the progress of growth e.g. height & weight measurements and sexual maturation characteristics. Chronological age, mental age, dental age, and skeletal age are some maturation indicators that have been used to identify stages of growth.[1]

Chronological age of the patient can be assessed by the date of birth. Maturational status of a child is governed by various factors like genetic, epigenetic, environmental, nutritional, hormonal etc[1] and hence chronological age has little importance in assessing skeletal maturity.[2]

Dental maturity can be estimated by the number of erupted and unerupted teeth, stages of dentition (deciduous, mixed or permanent); tooth calcification, degree of tooth structure, stages of crown formation of developing teeth and stages of root formation of all erupted teeth. Dental age being used as an indicator for maturity is a simple but not so accurate method because of wide variations in time of eruption of teeth due to the influence of local and environmental factors.[3]

Skeletal maturity assessment involves visual inspection of the developing bone and their initial appearance, sequential ossification, and related changes in shape and size. Thus, skeletal maturity indicators provide an objective diagnostic evaluation of stage of maturity in an individual. Skeletal growth and maturation are the outcome of complex interaction of many genes, hormones, growth factors and in addition environment. Lateral cephalometric radiographs are routinely taken for

orthodontic patients, using the cervical vertebrae from these radiographs to assess skeletal maturity has been especially appealing to orthodontists.[2] The risk of radiation exposure is the main concern with the use of lateral cephalogram as skeletal maturity indicator and also the evaluation is subjective, intra- examiner and inter-examiner bias is prone to happen.

Recent literature has given much emphasis on biochemical methods for detection of skeletal maturity. A biomarker is defined as "any substance, structure, or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease. Some of them are Serum Alkaline phosphatase, alkaline phosphatase in gingival crevicular fluid. Serum Insulin-like growth factor-1, DHEAS and Serum PTHrP have been used to explore their role in assessing the skeletal maturation of an individual.

Dehydroepiandrosterone (DHEA) and its sulfated conjugate dehydroepiandrosterone sulfate (DHEAS) are steroid hormones secreted from adrenal gland during adrenarche, which is a period of 3 years before puberty. They stimulate gonadostat (pituitary and hypothalamus together) to initiate puberty. In peripheral tissues, DHEA and DHEAS act as precursors of androgens and estrogens and hence, preventing the loss of androgens and estrogens into the circulation. They speed up growth and proliferation of epiphyseal cartilage and increase the GH activity.[4]

AIM:

Since radiographic methods are subjective and are prone to examination bias, the aim of this study is to co-relate plasma DHEAS and cervical vertebra maturation index, which can be useful in accurate determination of skeletal maturity avoiding radiation exposure.

MATERIALS AND METHOD

SOURCE OF DATA

- The subjects for the study were selected from the patients visiting Department of Orthodontics and Dentofacial Orthopedics, Dayananda Sagar College of Dental Sciences, Bengaluru coming for orthodontic treatment.

SAMPLE SIZE

- The sample size was calculated to be 90 individuals, both males and females between the age group of 8-18years.

STUDY DESIGN

Observational/Cross-Sectional study.

SAMPLE SIZE ESTIMATION

Analysis: A priori compute required sample size

Input: Effect size f = 0.40

α error prob	=	0.05
Power (1-β err pob)	=	0.80
Number of groups	=	6
Output: Noncentrality parameter	=14.4000000	
Critical F	= 2.3231265	
Numerator df	= 5	
Denominator df	= 84	
Total sample size	= 90	
Actual power	= 0.8225458	

The total sample was divided into 6 groups according to 6 CVM stages. Each group consisted of 15 samples. [15×6 groups =90 samples]. Importance was given to select 8 males and 7 females in each of the groups to check for gender based differences.

Criteria for case selection

INCLUSION CRITERIA

- Patients going to begin orthodontic treatment.
- Patients undergoing orthodontic treatment.
- Post treatment follow-up cases.
- Chronological age from 8-18yrs.
- No abnormal dental conditions such as impaction transposition and congenitally missing teeth.

EXCLUSION CRITERIA-

- Patients with systemic disease.
- Presence of growth abnormality or bleeding disorder.

- History of chronic medication.
- History of trauma or surgery in the area of cervical vertebrae.

Materials required:

- Syringe with needle-Dispo Van 2ml (Figure 1)
- Heparinized tubes-K2 EDTA (Figure 2)
- Deep freezer-Thermo (Figure 3)
- Centrifuging machine-Galaxy (Figure 4)
- Human DHEAS Elisa kit -DRG (Figure 5)
- Lateral cephalogram with X-ray viewer. (Figure 6)

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

METHODOLOGY

STEP 1: 6 groups were created from 90 subjects (Group 1- Group 6) with 15 in each group, according to the cervical vertebrae maturation (CVM) staging as mentioned by Baccetti, Franchi, and McNamara by

observation of cervical vertebrae C2, C3, C4 on the lateral cephalogram using an X-ray viewer Each subject was categorized in a particular CVM stage.

STEP 2: 2ml of random blood sample was collected by trained personnel from the subject. (Figure 1,2)

STEP 3: The sample was pipetted and stored in heparinized tube in a sealed plastic box and kept in a deep freezer at -20degree until assay. (Figure 4)

STEP 4: On the day of assay, sample was brought to room temperature and ultra-centrifuged. (Figure 4)

STEP 5: Measurement of serum DHEAS levels was made using human DHEAS-ELISA kits (Figure 5) which uses an in-vitro sandwich enzyme linked immunosorbent assay.

ASSAY PROCEDURE:

Prepare all reagents, working standards, and samples as directed in the previous sections.

Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, reseal and return to 4°C storage.

Add 30 µL standards, control or diluted samples into the respective wells. Add 100 µL DHEA sulfate-HRP Conjugate to each well. Leave a well empty as the substrate blank (without standards, control or diluted sample) and do not add the conjugate to this.

Cover wells with the foil supplied in the kit.

Incubate for 1 hour at 37°C.

When incubation has been completed, remove the foil, aspirate the content of the wells and wash each well three times with 300 µL diluted wash solution. Avoid overflows from the reaction wells. The soak time between each wash cycle should be > 5 seconds. At the end carefully remove remaining fluid by tapping strips on tissue paper prior to the next step.

Note: Washing is critical. Insufficient washing results in poor precision and falsely elevated absorbance values.

Add 100 µL TMB Substrate Solution into all wells.

Incubate for exactly 15 minutes at room temperature in the dark.

Add 100 µL Stop Solution into all wells in the same order and at the same rate as for the TMB Substrate Solution. Agitate the microplate gently using a rotating mixer. Any blue color developed during the incubation turns into yellow.

Measure the absorbance of the sample at 450 nm within 30 minutes of addition of the Stop Solution.

The same steps (STEP 1 to 5) were repeated to measure the Serum DHEAS level for each sample in all groups.

STATISTICAL ANALYSIS

Statistical analysis was done using software Gpower v 3.9.1.2. One way ANOVA test followed by Tukey's post hoc Analysis was used to compare mean DHEAS levels corresponding to the CV stages. Independent Student t-test was used to compare the mean serum DHEAS levels between genders in each study group. Significance level was set at $P<0.05$. Confidence interval was set at 95%.

RESULT

Table 1:

Comparison of mean levels of DHEAS [in pg/ml] between different CVM stages using One-way ANOVA Test						
Groups	**N**	**Mean**	**SD**	**Min**	**Max**	**P-Value**
CVM Stage 1	15	12.979	3.354	7.65	19.36	<0.001*
CVM Stage 2	15	17.647	6.160	11.31	27.37	
CVM Stage 3	15	32.112	3.726	26.04	37.56	
CVM Stage 4	15	42.582	8.797	32.25	57.16	
CVM Stage 5	15	68.347	8.261	54.69	80.24	
CVM Stage 6	15	76.279	9.602	61.11	90.31	

Comparison of mean levels of DHEAS between different CVM stages with one way ANOVA test. The mean levels of DHEAS between different CVM stages was statistically significant with p value <0.001.

Table 2:

Multiple comparison of mean difference in the DHEAS levels b/w different CVM stages using Tukey's Post hoc Analysis					
(I) Groups	**(J) Groups**	**Mean Diff. (I-J)**	**95% CI of the Diff.**		**P-Value**
			Lower	**Upper**	
CVM Stage 1	CVM Stage 2	-4.667	-12.209	2.874	0.47
	CVM Stage 3	-19.133	-26.674	-11.591	<0.001*
	CVM Stage 4	-29.603	-37.144	-22.061	<0.001*
	CVM Stage 5	-55.367	-62.909	-47.826	<0.001*
	CVM Stage 6	-63.299	-70.841	-55.758	<0.001*
CVM Stage 2	CVM Stage 3	-14.465	-22.007	-6.924	<0.001*
	CVM Stage 4	-24.935	-32.477	-17.394	<0.001*
	CVM Stage 5	-50.700	-58.242	-43.158	<0.001*
	CVM Stage 6	-58.632	-66.174	-51.090	<0.001*
CVM Stage 3	CVM Stage 4	-10.470	-18.012	-2.928	0.002*
	CVM Stage 5	-36.235	-43.776	-28.693	<0.001*
	CVM Stage 6	-44.167	-51.708	-36.625	<0.001*
CVM Stage 4	CVM Stage 4 CVM Stage 5	-25.765	-33.306	-18.223	<0.001*
	CVM Stage 6	-33.697	-41.238	-26.155	<0.001*
CVM Stage 5	CVM Stage 5 CVM Stage 6	-7.932	-15.474	-0.390	0.03*

Multiple comparison of mean difference in the DHEAS levels b/w different CVM stages using TUKEY,S post hoc analysis.

The multiple comparison between different CVM stages as shown in Table 2 demonstrates that mean DHEAS levels significantly increases from CVM stage 1 to stage 6, with majority of them significant at P<0.001. The mean increase in the DHEAS level from Stage 3 to Stage 4 was statistically significant at P=0.002 and between Stage 5 and Stage 6 at P=0.03. However no significant difference was found between CVM Stage 1 and Stage 2.

Table 3

Gender wise comparison of mean DHEAS levels [in pg/ml] in different CVM Stages using Independent Student t Test						
Groups	**Gender**	**N**	**Mean**	**SD**	**Mean Diff**	**P-Value**
CVM Stage 1	Males	8	15.46	2.28	5.32	<0.001*
	Females	7	10.14	1.60		
CVM Stage 2	Males	7	23.57	3.21	11.11	<0.001*
	Females	8	12.46	1.14		
CVM Stage 3	Males	8	34.97	1.97	6.12	<0.001*
	Females	7	28.85	2.14		
CVM Stage 4	Males	7	51.02	4.11	15.82	<0.001*
	Females	8	35.20	2.59		
CVM Stage 5	Males	8	74.81	4.62	13.85	<0.001*
	Females	7	60.96	3.87		
CVM Stage 6	Males	7	85.19	4.88	16.71	<0.001*
	Females	8	68.48	3.89		

Gender wise comparison of mean DHEAS levels in different CVM stages using independent student t test.

The results demonstrated that the mean DHEAS levels was significantly higher in males as compared to females in all CVM stages i.e. Stage 1 to Stage 6 with the p value <0.001.

Table 4

Comparison of mean levels of DHEAS [in pg/ml] between different CVM stages among Male subjects using One-way ANOVA Test						
Groups	**N**	**Mean**	**SD**	**Min**	**Max**	**P-Value**
CVM Stage 1	8	15.463	2.283	13.27	19.36	<0.001*
CVM Stage 2	7	23.570	3.207	18.74	27.37	
CVM Stage 3	8	34.968	1.967	31.59	37.56	
CVM Stage 4	7	51.021	4.111	46.64	57.16	
CVM Stage 5	8	74.811	4.616	65.68	80.24	
CVM Stage 6	7	85.190	4.876	78.54	90.31	

Mean levels of DHEAS were compared between different CVM stages among male subjects with the help of one way ANOVA test.

The results demonstrated that the mean levels of DHEAS between different CVM stages in male subjects was statistically significant.

Table 5

Multiple comparison of mean difference in the DHEAS levels b/w different CVM stages among Male subjects using Tukey's Post hoc Analysis					
(I) Groups	**(J) Groups**	**Mean Diff. (I-J)**	**95% CI of the Diff.**		**P-Value**
			Lower	**Upper**	
CVM Stage 1	CVM Stage 2	-8.108	-13.761	-2.454	0.001*
	CVM Stage 3	-19.505	-24.967	-14.043	<0.001*
	CVM Stage 4	-35.559	-41.213	-29.905	<0.001*
	CVM Stage 5	-59.349	-64.811	-53.887	<0.001*
	CVM Stage 6	-69.728	-75.381	-64.074	<0.001*
CVM Stage 2	CVM Stage 3	-11.398	-17.051	-5.744	<0.001*
	CVM Stage 4	-27.451	-33.291	-21.612	<0.001*
	CVM Stage 5	-51.241	-56.895	-45.588	<0.001*
	CVM Stage 6	-61.620	-67.459	-55.781	<0.001*
CVM Stage 3	CVM Stage 4	-16.054	-21.708	-10.400	<0.001*
	CVM Stage 5	-39.844	-45.306	-34.382	<0.001*
	CVM Stage 6	-50.223	-55.876	-44.569	<0.001*
CVM Stage 4	CVM Stage 5	-23.790	-29.443	-18.136	<0.001*
	CVM Stage 6	-34.169	-40.008	-28.330	<0.001*
CVM Stage 5	CVM Stage 6	-10.379	-16.032	-4.725	<0.001*

The multiple comparison of mean difference in the DHEAS levels between different CVM stages among male subjects as shown in Table 5 demonstrates that mean DHEAS levels significantly increases from CVM stage 1 to stage 6, with P value<0.001.

Table 6

Comparison of mean levels of DHEAS [in pg/ml] between different CVM stages among Female subjects using One-way ANOVA Test						
Groups	**N**	**Mean**	**SD**	**Min**	**Max**	**P-Value**
CVM Stage 1	7	10.141	1.595	7.65	12.36	<0.001*
CVM Stage 2	8	12.464	1.139	11.31	14.55	
CVM Stage 3	7	28.849	2.139	26.04	32.12	
CVM Stage 4	8	35.198	2.595	32.25	39.24	
CVM Stage 5	7	60.959	3.869	54.69	66.36	
CVM Stage 6	8	68.481	3.888	61.11	73.4	

Comparison of mean levels of DHEAS between different CVM stages among female subjects using one way ANOVA test.

The mean value of DHEAS levels between different CVM stages among female subjects is statistically significant.

Table 7

Multiple comparison of mean difference in the DHEAS levels b/w different CVM stages among Female subjects using Tukey's Post hoc Analysis					
(I) Groups	**(J) Groups**	**Mean Diff. (I-J)**	**95% CI of the Diff.**		**P-Value**
			Lower	**Upper**	
CVM Stage 1	CVM Stage 2	-2.322	-6.584	1.939	0.58
	CVM Stage 3	-18.707	-23.108	-14.306	<0.001*
	CVM Stage 4	-25.056	-29.317	-20.795	<0.001*
	CVM Stage 5	-50.817	-55.218	-46.416	<0.001*
	CVM Stage 6	-58.340	-62.601	-54.079	<0.001*
CVM Stage 2	CVM Stage 3	-16.385	-20.646	-12.124	<0.001*
	CVM Stage 4	-22.734	-26.850	-18.617	<0.001*
	CVM Stage 5	-48.495	-52.756	-44.234	<0.001*
	CVM Stage 6	-56.018	-60.134	-51.901	<0.001*
CVM Stage 3	CVM Stage 4	-6.349	-10.610	-2.088	0.001*
	CVM Stage 5	-32.110	-36.511	-27.709	<0.001*
	CVM Stage 6	-39.633	-43.894	-35.372	<0.001*
CVM Stage 4	CVM Stage 5	-25.761	-30.022	-21.500	<0.001*
	CVM Stage 6	-33.284	-37.400	-29.167	<0.001*
CVM Stage 5	CVM Stage 6	-7.523	-11.784	-3.262	<0.001*

The multiple comparison of mean difference in the DHEAS levels between different CVM stages among female subjects as shown in Table 7 demonstrates that mean DHEAS levels significantly increases from CVM stage 1 to stage 6, with majority of them significant at P<0.001. No statistically significant difference was found between CVM Stage 1 and Stage 2 with P=0.58.

DISCUSSION

There have been many studies done in the past to compare cranio-facial growth with skeletal maturity. Cervical vertebral maturation and hand-wrist radiographs are the most commonly used skeletal maturity indicators.

The Cervical Vertebral development given by McNamara, Baccetti and Franchi[5] uses morphology of second, third (C3) and the fourth (C4) cervical vertebrae. it involves examining the presence or absence of concavity at lower border of the body of C2, C3 & C4 and the shape of the body of C3 and C4.

Four fundamental shapes of C3 and C4 include Trapezoid, Rectangular, Squared and Rectangular vertical.

Studies done by McNamara et al[6] (1985), Petrovic et al[7] (1994) and Tulloch et al[8] (1997) demonstrated statistically and clinically significant correction of the Class II dentoskeletal relationships when either functional appliances or fixed appliances are used during the circumpubertal period. The CVM method can be helpful for the assessment of completion of active growth in studies dealing with the long term effects of orthodontic/orthopedic treatment strategies. Similarly, the method can be used to identify clinically the adequate time for intervention in subjects who need surgery for the late correction of facial disharmonies.

The disadvantages include difficulties in visualization of the subtle changes in the vertebrae, in visualization due to incorrect neck posture while taking the radiograph, blocking out of cervical vertebrae due to the use of a thyroid collar, inter-examiner bias and risk of radiation exposure.

Biomarkers have been used recently to find the skeletal maturity as they prevent radiation exposure. A biomarker is defined as "any substance, structure, or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease.

Some biomarkers used are alkaline phosphatase (ALP), Insulin like growth factor (IGF), Dehydroepiandrosterone sulphate (DHEAS), Parathyroid hormone related protein (PTHrP) of which all showed promise except PTHrP[3].

Dehydroepiandrosterone (DHEA) and Dehydroepiandrosterone sulphate (DHEAS) are steroid harmones produced by adrenal cortex of fetal adrenal glands. It decreases to negligible amounts at birth and the levels are less until 5- 7 years of age. The steroid level reaches its peak at the age of 20-30 years[9,10].

According to Hopper and Yen [10] (1975); Sulcova et al [11] (1984) Serum levels of DHEAS are high in the neonate, after which there is a decrease, then a rapid increase in the serum level from 7 years of age in females and 8 years of age in males, with a gradual increase until it attains the adult value.

DHEA(S) is an androgen related with development. According to Clare Netherton et al [12], Robert Matchock et al [13] and Wayne Meikle et al [14] have confirmed the relation of DHEA and DHEAS with pubertal growth.

According to Nele Friedrich et al [15] DHEA levels follow a circadian rhythm with a peak in the morning, DHEAS levels are relatively stable throughout the day and thereby is used as markers for DHEA levels and adrenal androgen secretion. Lesser quantity of salivary DHEAS was found by Vining et al [16] because of bigger molecular size.

In our investigation, there is a dynamic increase in serum DHEAS level as skeletal development advanced from CVMI stages 1 and 2, CVMI stages 3 and 4 arriving at the most noteworthy amplification at CVMI stages 5 and 6.

The mean level of DHEAS was 12.97±3.35pg/ml at CVM stage 1, 17.64±6.1pg/ml at CVM stage 2, 32.1±3.37pg/ml at CVM stage 3, 42.58±8.79pg/ml at CVM stage 4, 68.34±8.26pg/ml at CVM stage 5 and 76.27±9.60pg/ml at CVM stage 6. The

values are statistically significant with P value <0.001.

Multiple comparison between different CVM stages demonstrates that mean DHEAS levels significantly increases from CVM stage 1 to stage 6, with majority of them significant at P<0.001. The mean increase in the DHEAS level from Stage 3 to Stage 4 was statistically significant at P=0.002 and between Stage 5 and Stage 6 at P=0.03. However, no significant difference was found between CVM Stage 1 and Stage 2 [P=0.47].

The results of our study are in concurrence with the investigation by Clare Netherton et al. [12] R.L. Matchock et al [13] that related the serum DHEAS levels of subjects and their pubertal status based on Tanner staging[79] and noted higher mean levels in mid-post pubertal young male and young females than in early pubertal male and female individuals.

According to our study the mean DHEAS serum level is more in males comparatively in females, which is as a result of adrenal corticotrophin secretion.

This is as per the study done by Young et al [17,18] who noticed mean levels of DHEA-S in all age groups were significantly higher in males.

The advantages of using serum DHEAS for assessment of skeletal maturity is accuracy over other radiographic techniques. Inter-examiner and intra-examiner variations are ruled out, which is seen with the radiographic techniques. Additional radiographic exposure to the patient is avoided, using this technique.

The disadvantages include difficulties in storage and transport of the samples to the laboratory for analysis. The patient cooperation for collection of samples among the younger children is difficult. Also this technique is not very cost effective.

CONCLUSION

- The above study was done to co-relate serum dehydroepiandrosterone levels and cervical vertebral maturation.
- The conclusion of the study is as follows:

1. There was statistically significant difference in the mean serum levels of dehydroepiandrosterone sulfate (DHEAS) in six CVM stages. And between males and females also.
2. The correlation between the two shows that the serum DHEAS can be a possible indicator of skeletal maturation. Longitudinal data are needed to confirm the usefulness of this technique to accurately determine whether serum DHEAS levels are good predictors of skeletal maturity.

List Of Abbreviations:

Abbreviations	Expanded
CVMI	Cervical vertebrae maturational indicator
PHV	Peak height velocity
OPG	Orthopantomogram
PTHrP	Parathyroid hormone receptor protein
IGF	Insulin like growth factor
IGFBP	Insulin like growth factor binding protein
DHEAS	Dehydroepiandrosterone sulfate
GH	Growth hormone
ELISA	Enzyme linked immunosorbent assay
HPA	Hypothalamic pituitary adrenal axis
MPO	Myeloperoxidase
LDH	Lactate Dehydrogenase
GCF	Gingival Crevicular Fluid
ALP	Alkaline Phosphatase

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