ORIGINAL ARTICLE

DOI: 10.4274/jcrpe.galenos.2023.2023-5-22

J Clin Res Pediatr Endocrinol 2024;16(1):84-90

Effect of Adrenocorticotropic Hormone Stimulation on Ischemia-modified Albumin Levels *in vivo*

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What is already known on this topic?

Ischemia-modified albumin (IMA) levels are positively correlated with reactive oxygen species (ROS). It is known that cortisol leads to decreased ROS production. The effect of adrenocorticotropic hormone (ACTH) stimulation on IMA levels is not known.

What this study adds?

We found that standard-dose ACTH stimulation rapidly reduces levels of IMA *in vivo*. It is possible that the relationship between ACTH and IMA is dose-dependent and ACTH has a direct effect on IMA.

Abstract

Objective: Ischemia-modified albumin (IMA) formation is associated with increased reactive oxygen species (ROS) production, while increased cortisol leads to decreased ROS levels. We aimed to evaluate the effect of adrenocorticotropic hormone (ACTH) stimulation on IMA levels and whether the effect was dose-dependent or not.

Methods: A total of 99 subjects with normal ACTH test results were included in the study. Of these, 80 had standard-dose ACTH test while 19 had low-dose ACTH test. Blood samples were collected to determine cortisol and IMA levels; at minutes 0, 30, and 60 following the standard-dose ACTH test and at minutes 0 and 30 following the low-dose ACTH test.

Results: IMA levels decreased significantly within 30 minutes and the decrease continued up to the sixtieth minute ($p = 0.002$) after standard-dose ACTH stimulation. After ACTH stimulation, a weak negative correlation was found between peak cortisol and IMA levels at the thirtieth minute ($r = 0.233$, $p = 0.02$). There was no significant difference in IMA levels after low-dose ACTH stimulation, despite an increase in cortisol ($p = 0.161$).

Conclusion: IMA levels decreased rapidly after standard-dose ACTH stimulation, while a decrease in IMA levels was not observed after low-dose ACTH stimulation. The lack of decrease in IMA levels after low-dose ACTH stimulation suggests a possible dose-dependent relationship between ACTH and IMA. The moderate increase in cortisol with no reduction in IMA levels after low-dose ACTH stimulation and the weak correlation between peak cortisol and 30-minute IMA levels after standard-dose ACTH stimulation suggest that ACTH may have a direct effect on IMA.

Keywords: Ischemia-modified albumin, ACTH, cortisol, reactive oxygen species

Presented in: The content of the manuscript was presented at the European Society for Paediatric Endocrinology (ESPE) 2022 in Rome (Free communication).

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Conflict of interest: None declared
Received: 27.07.2023
Accepted: 06.11.2023

Introduction

Albumin is synthesized in the liver and contains three homologous domains, each consisting of two subdomains (1,2). Albumin contains Sudlow sites 1 and 2, which play an important role in the transport of hydrophobic molecules, heme binding sites, small ligand binding sites, seven fatty acid binding sites, and four metal binding sites, including the sites A and B, N-terminal site (NTS) and Cys34 (2,3,4). Metal binding sites have different affinity for each metal. NTS has the highest affinity for copper, nickel, and cobalt (3). The NTS of albumin is very susceptible to biochemical alteration and degradation.

Reactive oxygen species (ROS) play a role in regulating signaling pathways but must be kept in limited amounts in the body because ROS cause oxidative damage to DNA, RNA, proteins, and lipids (5). Oxidative stress occurs when ROS production exceeds the capacity of antioxidant mechanisms to neutralize ROS (5). Dipeptide cleavage occurs in the NTS of albumin due to increased ROS during oxidative stress (6). The truncated NTS cannot bind metal ions (7). This variant of albumin is called ischemia-modified albumin (IMA). IMA production increases during oxidative stress due to excessive ROS formation (6). Moreover, excessive ROS production has also been shown to play a role in the etiology of various inflammatory diseases through the induction of inflammation (8,9). Therefore, IMA is considered a parameter for the assessmentof both oxidative stress and inflammation.

Cortisol exerts anti-inflammatory effects through genomic and non-genomic pathways, resulting in decreased cytokine and ROS production (10). Adrenocorticotropic hormone (ACTH) has an anti-inflammatory effect through a glucocorticoid-dependent pathway by stimulating endogenous cortisol release. However, the anti-inflammatory effects of ACTH were preserved in adrenalectomized rats (11). ACTH also has a glucocorticoid-independent anti-inflammatory effect by activating melanocortin receptors expressed on immune cells (11). While IMA production increases with excessive ROS formation, ACTH and cortisol cause ROS production to decrease (6,10,11). However, the effect of *in vivo* administration of ACTH, which stimulates endogenous cortisol release, on IMA levels is unknown. The aim of this study was to determine whether ACTH stimulation has an impact on IMA levels and, if so, whether the effect is dependent on the dosage.

Methods

Between February 2021 and February 2022, children who would undergo standard-dose ACTH testing with suspicion of congenital adrenal hyperplasia and children who would undergo low-dose ACTH testing with suspicion of central adrenal insufficiency at a single center were included in the study. Subjects with acute or chronic diseases, active infections, obesity, or drug use were excluded. All participants and/or parents were informed orally and in writing and consented to participate. The study is in accordance with the World Medical Association Declaration of Helsinki and was approved by the University of Health Sciences Turkey, Dr. Sami Ulus Pediatric Health and Disease and Obstetrics and Gynecology Training and Research Hospital Local Ethics Committee (project no: E-21/01-76, date: 21.01.2021).

All subjects were outpatients during the ACTH test. Physical exam and pubertal staging were conducted. For the standard-dose ACTH test, children under two years of age were given 125 mcg of intravenous tetracosactide, while those over two years of age were given 250 mcg. Blood samples were collected during the standard-dose ACTH test at 0, 30, and 60 minutes to measure cortisol and IMA levels, between 08:00 and 09:00 in the morning. Subjects with abnormal standard-dose ACTH stimulation test results were excluded from the study, and 80 subjects who underwent standard dose ACTH tests were included in the study. In the low-dose ACTH test, all subjects were given 1 mcg of tetracosactide. During the low-dose ACTH test, blood samples were taken at 0 and 30 minutes to assess cortisol and IMA levels. Subjects with low-dose ACTH stimulation test results compatible with adrenal insufficiency were excluded from the study. Nineteen subjects who underwent low-dose ACTH tests and had normal results were included in the study. In total, 99 healthy subjects participated in the study.

Chemiluminescent immunoassay was used to analyze serum cortisol concentrations (Siemens Advia XPT). ACTH was analyzed by chemiluminescent immunoassay method (Siemens Immulite 2000 XPi). Blood samples were centrifuged at 3500 rpm for 5 minutes and stored at -80 °C until the IMA test was performed. The albumin cobalt binding test was used to analyze serum IMA concentrations (12). The test procedure is as follows: 50 µL of 0.1% cobalt (II) chloride ($CoCl_2$, $6H_2O$) (Sigma-Aldrich Chemie GmbH, Riedstrasse 2, Steinheim, Germany) was added to 200 µL of subject serum. After mixing, followed by 10 minutes of incubation to allow for albumin cobalt binding, 50 µL 1.5 mg/mL dithiothreitol was added, mixed and incubated for 2 minutes at body temperature. Then 1.0 mL of a 0.9% sodium chloride solution was added in order to reduce the binding capacity. The blank was prepared with distilled water without dithiothreitol. The absorbance of the samples was measured at 470 nm with a spectrophotometer (12) and given as absorbance units.

Statistical Analysis

All statistical analyses were performed using the Statistical Package for Social Sciences software, version 17 (IBM Inc., Chicago, IL, USA). Non-parametric tests were used after the normal distribution conformity test. Qualitative data were expressed as numbers/percentage and quantitative data as median (range). The Mann-Whitney U test was used to compare the two groups. To determine the relationship between two continuous variables, the Spearman's correlation coefficient was used to. A statistically significant p value was considered as < 0.05.

Results

The basal ACTH, cortisol, and IMA levels of subjects are given in Table 1. There was no correlation between IMA levels and age of subjects ($p > 0.05$). Basal IMA levels of boys and girls did not differ ($p > 0.05$). More than half (50.5%) of the subjects were pubertal, and the basal and 30th-minute IMA values of the pubertal subjects did not differ from those of the prepubertal subjects ($p = 0.656$ and $p = 0.768$, respectively). There was no correlation found between body mass index (BMI) standard deviation score (SDS) and basal IMA, as well as BMI SDS and 30-minute IMA levels ($r = -0.049$, $p = 0.632$ and $r = -0.129$, $p = 0.204$ respectively). There was no correlation between basal ACTH and IMA as well as basal cortisol and IMA levels ($p > 0.05$).

The results of standard-dose ACTH stimulation: The median age of 80 subjects who underwent standard-dose ACTH test was 5.5 (0.66-17) years. The stimulated cortisol and IMA levels of subjects are given in Table 1. IMA levels decreased significantly within 30 minutes and the decrease continued to 60 minutes after standard-dose ACTH stimulation ($p = 0.002$) (Figure 1). IMA levels at 30 and 60 minutes were similar ($p = 0.773$). There was a positive correlation between basal and 30 as well as basal and 60-minute IMA levels ($r = 0.675$, $p = 0.0001$ and $r = 0.676$, $p = 0.0001$ respectively) (Figure 2). There was a weak negative correlation between peak cortisol and IMA levels 30 minutes after ACTH stimulation ($r = 0.233$, $p = 0.02$) (Figure 3). Basal and stimulated IMA levels did not differ according to gender ($p > 0.05$).

Low-dose ACTH stimulation: Nineteen subjects with a median age of 9.2 (0.1-17.8) years underwent low-dose ACTH test. The stimulated cortisol and IMA levels of subjects are given in Table 1. No significant difference was observed in IMA levels after low-dose ACTH stimulation ($p = 0.161$) (Figure 4). There was no correlation between peak cortisol and IMA levels at the thirtieth minute ($r = 0.103$, $p = 0.667$) (Figure 5). Basal and stimulated IMA levels were not different according to gender ($p > 0.05$).

Comparison of low-dose and standard-dose ACTH stimulation: Basal ACTH and IMA levels were similar in the two groups ($p = 0.353$ and $p = 0.147$, respectively) (Table 1). Although there was a difference between basal cortisol levels, there was no correlation between basal cortisol and basal IMA levels between the two groups ($r = -0.102$, $p = 0.315$). In subjects given standard-dose ACTH stimulation, basal and peak cortisol levels were higher and IMA levels at the thirtieth minute were significantly lower ($p = 0.003$, $p = 0.00001$, and $p = 0.002$ respectively) (Table 1).

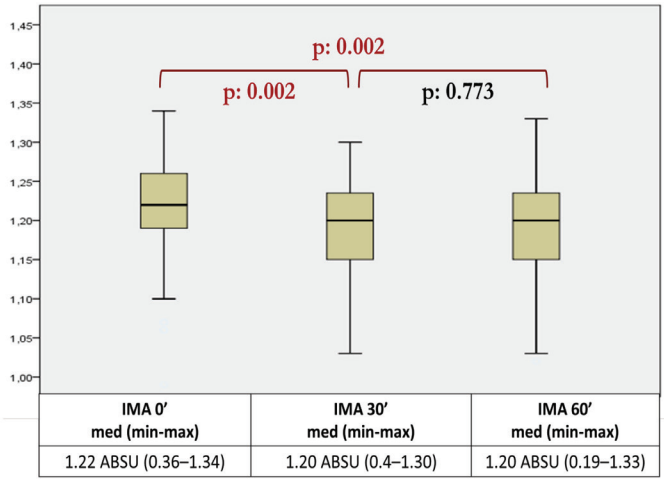

Figure 1. IMA levels after standard-dose ACTH stimulation

IMA: ischemia-modified albumin, ACTH: adrenocorticotropic hormone, Min-max: minimum-maximum, ABSU: absorbance units

Table 1. Comparison of low-dose and standard-dose ACTH stimulation responses

	Low-dose ACTH test			Standard-dose ACTH test			p
	Med	Min	Max	Med	Min	Max	
Basal ACTH pg/mL (pmol/L)	18.4 (4.1)	8.7 (1.9)	43.7 (9.6)	17.7 (3.9)	5 (1.1)	68 (15)	0.353
Basal cortisol mcg/dL (nmol/L)	5.3 (146.3)	1.9 (52.4)	16.8 (463.7)	12 (331.2)	4.1 (113.2)	35.3 (974.3)	0.003
Peak cortisol mcg/dL (nmol/L)	22.5 (621)	18.1 (499.6)	27.7 (750.7)	31.8 (877.7)	19.2 (529.9)	41.9 (1156.4)	0.0001
IMA 0' minute (ABSU)	1.25	0.94	1.29	1.22	0.36	1.34	0.147
IMA 30' minute (ABSU)	1.25	0.96	1.44	1.20	0.40	1.30	**0.002**

ACTH: adrenocorticotropic hormone, IMA: ischemia-modified albumin, Min: minimum, Max: maximum, ABSU: absorbance units

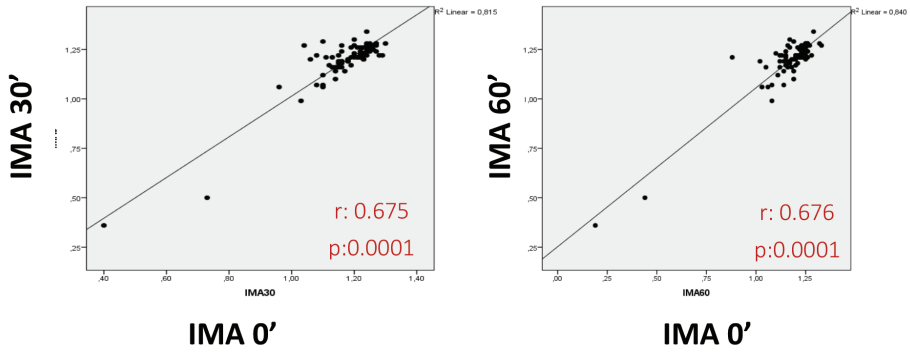

Figure 2. The correlation of basal to 30 and basal to 60 minutes IMA levels after standard-dose ACTH stimulation

IMA: ischemia-modified albumin, ACTH: adrenocorticotropic hormone

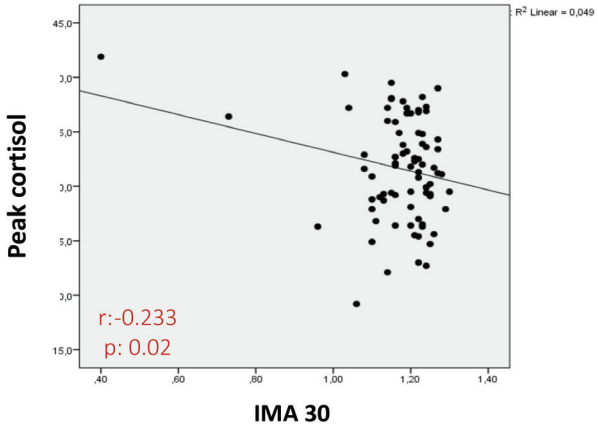

Figure 3. The correlation of peak cortisol and IMA levels at 30 minutes after standard-dose ACTH stimulation

IMA: ischemia-modified albumin, ACTH: adrenocorticotropic hormone

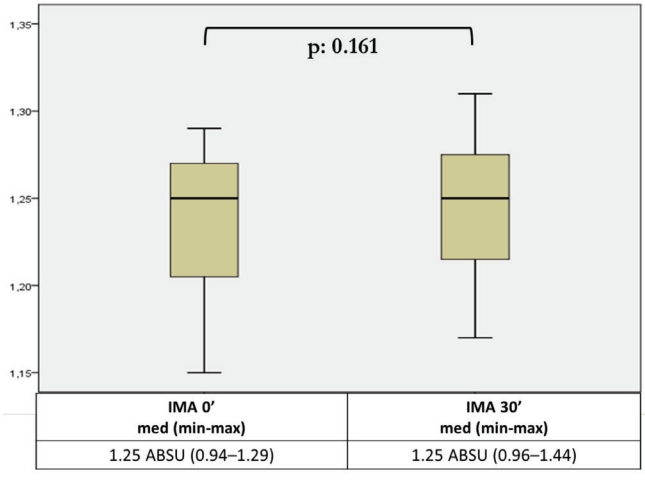

Figure 4. IMA levels after low-dose ACTH stimulation

IMA: ischemia-modified albumin, ACTH: adrenocorticotropic hormone, Min-max: minimum-maximum, ABSU: absorbance units

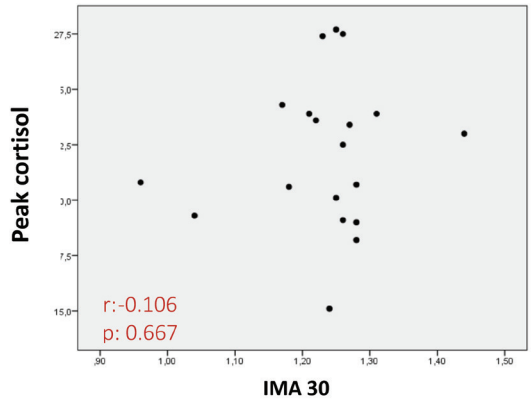

Figure 5. The correlation of peak cortisol and IMA levels at 30 minutes after low-dose ACTH stimulation

IMA: ischemia-modified albumin, ACTH: adrenocorticotropic hormone

Discussion

To the best of our knowledge, this is the first study to evaluate the effect of ACTH stimulation on IMA levels. It is known that increased ROS production during oxidative stress leads to protein oxidation and the production of inflammatory signals, contributing to the development of several chronic diseases (8,9,13,14,15). It has also been shown that IMA formation is associated with increased ROS production and inflammation in oxidative stress and IMA levels rise 6-10 minutes after oxidative stress (6,16,17). However, the alteration of albumin to IMA is reversible, and IMA rapidly returns to its basal level after ischemia (18). In the present study, it was observed that standard dose ACTH stimulation reduced IMA levels *in vivo* at 30 minutes after stimulation. This effect of ACTH on IMA may occur through stimulation of cortisol secretion by ACTH. It has been reported that cortisol strengthens antioxidant defenses by increasing the reduced form of glutathione, thus limiting the production of pro-oxidants such as ROS (19,20). Since cortisol reduces

ROS levels, it is expected to reduce IMA formation. Most glucocorticoid effects are due to the transcriptional effects of the glucocorticoid receptor, which represses the transcription of pro-inflammatory cytokine and chemokine genes (21). However, these mechanisms might not be involved in the more rapid anti-inflammatory effects of glucocorticoids. Studies have shown that glucocorticoids have rapid, non-genomic effects on ROS formation via the generation of intracellular second messengers and signal transduction cascades (22,23,24). In the present study, the rapid decrease in IMA levels after standard-dose ACTH stimulation suggests that cortisol exerts its effect on IMA by a non-genomic pathway. After administering standard-dose ACTH stimulation, we observed a statistically significant but relatively small difference in IMA levels. As it is widely known, high levels of IMA are found in various diseases that are associated with ischemia and oxidative stress. In such cases, ACTH stimulation may lead to a significant decrease in IMA levels. However, it is important to note that our study population comprised healthy individuals. Therefore, the alteration in IMA levels that we observed was relatively low.

IMA levels were also evaluated during a low-dose ACTH test to observe the effect of the ACTH stimulation dose.The lack of a decrease in IMA levels after low-dose ACTH stimulation suggests that a possible dose-dependent relationship between ACTH and IMA. However, the presence of only 19 subjects in the low-dose ACTH test group may have prevented a statistically significant result.

It is known that ACTH is more effective than corticosteroids in treating some inflammatory diseases. Getting et al. (25) demonstrated that local ACTH injection has remarkable anti-inflammatory effects. The same group demonstrated that the anti-inflammatory effects of ACTH were preserved in adrenalectomized rats (25). They also demonstrated that the 4-10th amino acid-containing parts of ACTH cannot stimulate cortisol secretion but can inhibit macrophage activation and neutrophil accumulation in inflammatory exudates (26). In summary, while it is known that ACTH has anti-inflammatory effects other than stimulating cortisol secretion, there is no information that ACTH directly affects ROS and IMA levels. In our study, although standard-dose ACTH stimulation provided a remarkable increase in cortisol and decrease in IMA, there was a weak negative correlation between peak cortisol and IMA levels 30 minutes after ACTH stimulation. There was no significant difference in IMA levels after low-dose ACTH stimulation, despite providing a moderate increase in cortisol. These results indicate that high doses of ACTH may have a direct effect on ROS and IMA levels.

In the present study, in which the effect of ACTH stimulation on IMA levels was evaluated, although the basal IMA and basal ACTH values of the two groups were not different, there was a significant age difference between the two groups. However, we found no correlation between IMA levels and the age of subjects. Moreover, there is no evidence in the literature that suggests IMA changes with age. Since the basal IMA and ACTH level of the two groups is not different we think that age will not be expected to have an effect on the IMA level after ACTH stimulation.

It has been found in some studies that the IMA/albumin ratio should be evaluated alongside IMA levels. A recent review explains that the total plasma albumin concentration should be taken into account, especially in individuals with hypo- or hyperalbuminemia, to avoid misinterpretation of IMA values (27). This is particularly important when examining the level of IMA in other biological fluids such as urine or saliva (27). The study evaluated IMA levels in the blood of healthy participants. It is important to note that since the participants were healthy, it is unlikely that their blood albumin levels would be abnormal.

Study Limitations

The main limitation of our study was the lack of post-stimulation ACTH level measurements. According to the protocol used in our clinic, the amount of standard dose ACTH test tetracosactide is determined according to age, 125 mcg was given to subjects under 2 years of age, and 250 mcg was given to subjects over the age of 2. In contrast, in the low-dose ACTH test, 1 mcg tetracosactide was given to all subjects. Due to the situation, the dosage of ACTH given to the subjects varied based on micrograms per kilogram. It is possible that after administering standard and low doses of ACTH stimulation, the level of ACTH in the blood may have varied among the subjects, which could have influenced our results. However, the lack of correlation between baseline ACTH and IMA suggests that possible individual differences in blood ACTH levels may have a limited effect on the results. The second limitation of our study was that there were only 19 subjects in the low-dose ACTH test group, which may not be sufficient to obtain a statistically significant result.

Conclusion

The effect of ACTH stimulation on IMA was evaluated forthe first timein this study. It was found that standard-dose ACTH stimulation reduced levels of IMA at 30 and 60 minutes after ACTH stimulation *in vivo*, while a decrease is not observed after low-dose ACTH stimulation. The lack of decrease in IMA levels after low-dose ACTH stimulation suggests a

possible dose-dependent relationship between ACTH and IMA. The moderate increase in cortisol with no reduction in IMA levels after low-dose ACTH stimulation and the weak correlation between peak cortisol and thirtieth minute IMA levels after standard-dose ACTH stimulation suggest that ACTH may have some direct effects on IMA levels other than stimulating cortisol secretion. Clarifying functional mechanisms will help understand the pathogenesis of diseases and develop treatments.

Acknowledgement

We would like to thank Fatma Budak for doing the tests and had wonderful cooperation with the children during sampling.

Ethics

Ethics Committee Approval: The study is in accordance with the World Medical Association Declaration of Helsinki and was approved by the University of Health Sciences Turkey, Dr. Sami Ulus Pediatric Health and Disease and Obstetrics and Gynecology Training and Research Hospital Local Ethics Committee (project no: E-21/01-76, date: 21.01.2021).

Informed Consent: All participants and/or parents were informed orally and in writing and consented to participate.

Authorship Contributions

Surgical and Medical Practices: Nursel Muratoğlu Şahin, Senem Esen, Şenay Savaş Erdeve, Semra Çetinkaya, Concept: Nursel Muratoğlu Şahin, Şenay Savaş Erdeve, Salim Neşelioğlu, Özcan Erel, Semra Çetinkaya, Design: Nursel Muratoğlu Şahin, Senem Esen, Data Collection or Processing: Nursel Muratoğlu Şahin, Senem Esen, Şenay Savaş Erdeve, Salim Neşelioğlu, Özcan Erel, Semra Çetinkaya, Analysis or Interpretation: Nursel Muratoğlu Şahin, Şenay Savaş Erdeve, Semra Çetinkaya, Literature Search: Nursel Muratoğlu Şahin, Writing: Nursel Muratoğlu Şahin, Şenay Savaş Erdeve, Semra Çetinkaya.

Financial Disclosure: The authors declared that this study received no financial support.

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