Disturbances of Amino Acid Metabolism in Neurologic Disorders detected by fluorescent high performance liquid chromotograghy (HPLC) in Baghdad - IRAQ

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ABSTRACT

Background:Amino acid disorders are a major group of inborn error metabolism (IEM) with variable clinical presentation; its diagnosis constitutes a real challenge in a community with high consanguinity rate and no systematic newborn screening.

Objectives: to provide data about amino acid disorders detected in high-risk Iraqi children by using quantitative amino acid fluorescent high performance liquid chromatography (HPLC) analysis.

Type of the study: Cross-sectional study.

Methods: a descriptive cross sectional study from 1st February to 1st December 2014, at Neurological ward and clinic of the Children Welfare teaching Hospital, in Baghdad - Iraq. Plasma specimens of 500 patients, with clinical suspicion of inborn error of metabolism (IEM) because of unexplained neurological deficits, unexplained developmental delay, recurrent coma and/or Neuro-degeneration, hair changes and/or lethargy, poor feeding, vomiting and selected cases of autistic spectrum syndrome or with positive screening, were analyzed for amino acids by high performance liquid chromatography (HPLC). The amino acid disorders were confirmed in fifty patients were; clinical data of patients were reported and analyzed statistically.

Results: out of 500 patients visiting the neurological outpatient and ward, clinical and neurological finding were recorded as well as the family history and/or other symptoms suggestive of aminoacidopathy, Sixty patients were confirmed their diagnosis as amino acid disorders, ten patients were excluded because they lost the follow up or there is no solid base for a causal relationship between detected abnormal amino acids and neurological disorders, therefore only 50 patients were enrolled in the study. Patients with Phenylketonuria were the most frequent 24 (48%), homocystinuria 14 (28%), maple syrup urine disease 9 (18%) & other amino acid disorder, (Citrullinemia, non-ketotic hyperglycinemia & Tyrosinemia) 1for each disorder (2%). Considerable delay in diagnosis was noticed which lead to variable neurological abnormalities in most patients and the psychomotor delay was the main clinical presentation at time of diagnosis 34 (68%).

Conclusion: in the absence of newborn screening, the majority of Aminoacidopathies cases was still diagnosed clinically, but delayed. The importance of clinical awareness and accurate biochemical analysis were the key tools for diagnosis and the necessity for a comprehensive national newborn screening program.

Keywords; HPLC,aminoacidopathy, metabolism .

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Typical Aminoacidopathies result from abnormalities in the breakdown of amino acids in the cytosol. In addition, several disorders involving mitochondrial enzymes such as branched-chain ketoacid dehydrogenase (maple syrup urine disease (MSUD)) or ornithine aminotransferase (gyrate atrophy of the choroidea) are classified as Aminoacidopathies as they do not involve CoA-activated metabolites; this distinguishes Aminoacidopathies from the organic acidurias, which are considered a separate group of disorders affecting mitochondrial enzymes, CoA-activated metabolites, and which have effects on other mitochondrial function. [1] Clinical symptoms of the Aminoacidopathies may be thought as caused by the accumulation of toxic intermediates that cause specific organ damage. Several defects of amino acid metabolism, such as histidinemia are benign because the metabolites that accumulate are not toxic. Aminoacidopathies are diagnosed through the analysis of plasma (or urine), concentrations of amino acids and sometimes of urinary organic acids. [1]

The majority of Aminoacidopathies are treatable through dietary restriction of protein and of the amino acid involved in the defective pathway and by the avoidance or prompt treatment of catabolic states that lead to the breakdown of large amounts of protein.

Another therapeutic strategy that has been successful in hepatorenal Tyrosinemia is the inhibition of a biochemical step before the actual genetic deficiency, thereby changing a harmful disease into a more benign amino acid accumulation without the accumulation of the more damaging substances downstream. [1]

The biochemical hallmarks of these upsets can be detected by sophisticated laboratory method such as high performance liquid chromatography (HPLC) for amino acids. HPLC is a tool used for keying out, quantifying, separating and purifying chemical compounds. It is usually practiced in the research labs of food manufacturing, pharmaceutical, mining and hmedical companies for quality control and inquiry. [2]

The concept of inborn error of metabolism was introduced by Archibald Garrod in the early 20th century through his documentation of four conditions, namely albinism, alkaptonuria, cystinuria, & benign pentosuria that were due to enzyme deficiencies inherited in an autosomal recessive manner. [3] The biochemical hallmarks of these disorders can be detected by sophisticated laboratory method such as high performance liquid chromatography (HPLC) for amino acids. [4]

HPLC, called high performance liquid chromatography or high pressure liquid chromatography, is a tool used for identifying, quantifying, separating and purifying chemical compounds. It is commonly used in the laboratories of food manufacturing, pharmaceutical, mining and medical companies for quality control and research. In order to use an HPLC properly, it is important to understand the function and purpose of its basic components.

Liquid chromatography was defined in the early 1900s by the work of the Russian botanist, Mikhail S. Tswett. His pioneering studies focused on separating compounds [leaf pigments], extracted from plants using a solvent, and in a column packed with particles. [5]

Identification and diagnosis of such disorders remain a real challenge in Iraq, however ministry of health adopted a program for neonatal screening that restricted to three Inborn errors of metabolism (IEM), these are Phenylketonurea (PKU), hypothyroidism and galactossemia. So we try to provide data about amino acid disorders detected in high-risk Iraqi children by using quantitative amino acid fluorescent high performance liquid chromatography (HPLC) analysis.

Methods:

Setting: A single center descriptive cross sectional study was conducted at the pediatric neurology department and clinic at the Welfare Teaching Hospital, Medical City, Baghdad-Iraq, for the period from 1st February to 1st December 2014. A total of 500 patients, who referred to a fluorescent HPLC analysis for suspected Aminoacidopathies, in addition to the basic metabolic investigation. Most of patients are well known to the neurological service by their Psychomotor delay & behavioral abnormalities who were undiagnosed.

Ethical approval: This study was performed according to the Helsinki II Declaration and approved by the ethics committee at the Welfare Teaching Hospital, Medical City. All patient's parents were informed about the aim and the suspected benefit of the study before obtaining their agreements for participation; a verbal consent was obtained from all parents.

All the medical research ethics rules and instructions regarding patient's privacy, humanity and security; as well as the medical research, laboratory data and investigation results were strictly considered throughout all the steps of the study.

The inclusion criteria : The ages of all patients enrolled in the study were between 2months and 15 years with the following criteria :

1. Patients who already well known to neurological clinic with intractable seizures unclassified with the syndrome, unexplained neurological deficits, unexplained developmental delay, recurrent coma, neurodegeneration, ophthalmologic findings suggestive of IEM, selected cases of autistic spectrum disorder with positive family history and/or unusual urine odor, altered mental status out of proportion of other systemic disorder unusual body or urine odor and hiccup.
2. The abnormal laboratory findings included persistent and/or recurrent hypoglycemia, metabolic acidosis with increased anion gap, hyperammonemia, cytopenia, imaging suggestive of IEM and abnormal newborn screening for PKU.

The exclusion criteria included;

1. Age: below 2 months and above 15 years
2. Uncertain clinical relevance between abnormal amino acid elevation and the clinical presentatio.

Out of 500 patients, 60 patients were found to have an amino acid(AA) disorder and 10 patients were excluded because of withdrawal from study for different reasons; one patient with phynelketonuria (PKU), four patients with increased prolin AA, three with increase Histidine, one with increase lysine and one with increase tyrosin. The one with PKU had been excluded because loss of follow up while those with hyperprolinemia (whether type 1 or 2) ,neither types causes any specific clinical manifestation[6] ,and also same for Histidine is degraded through the urocanic acid pathway to glutamic acid ,Several genetic conditions involving the degeradative pathway of Histidine have been reported but none has any clinical consequence [6]. Regarding increased tyrosine, Hypertyrosinemia is a common artifact in blood samples obtained soon after eating [6] and his clinical synthase (AASS gene);uncertain clinical relevance [7]. Therefore 50 patients enrolled feature not suggestive and patient not repeat the test fasting, while hyperlysinaenemia rare deficiency of 2-aminoadipic semialdehyde in the study based on a suggestive clinical presentation in addition to HPLC prove diagnosis,Therefore 50 patients enrolled in the study based on a suggestive clinical presentation in addition to HPLC proved diagnosis.

Study Participants: Participants were allocated to four groups, hemocystinurea (HCU) group (n=14), maple

syrup urine disorder (MSUD) group (n=9), phynelketonuria (PKU) group (n=24) and other disorders group (n=3). They were thoroughly interviewed, clinically examined and investigated according to the approved standards medical and laboratory work up.

Patients diagnosed with each specific AA disorder were categorized according to the presenting clinical signs, demographic information and consanguinity among patients' families, severity of disease and compare with level AA, and various transitions and the calculation of the quality of each analyst determined and analyzed in relation one to other AA.

Basic diagnostic investigations: Up on clinical suspicion of an AA disorder, basic metabolic investigations were done using routine methods for measurement of serum glucose, electrolyte liver function test (aspartate aminotransferase, alanine amino transferase). Arterial blood gases, ammonia, lactate and selected cases need advanced diagnostic biochemical investigations carried out for diagnosis AA disorder, for instance non ketotic hyperglycinemia(NKH) we do CSF glycin.

Definitions: Psychomotor retardation or developmental delay; refers to the slow progress in attainment of developmental milestones this may be caused by either static or progressive encephalopathies. [8] Intellectual disability; the term intellectual disability is replacing mental retardation ,defines as a disability characterized by a significant limitation both intellectual functioning and in adaptive behavior as expressed in conceptual ,social ,practical and adaptive skills .this disability originate before the age of 18 and manifest with severe problems in the individuals capacity to perform (i.e. impairment), ability to perform (i.e. activity limitation),and opportunity to function (participation restrictions) [9]

A cousins; is a relative with whom a person share one or more common ancestor, in general sense , cousins are two or more generations away from any common ancestor

First cousins: the children of two siblings . first cousins have grandparents in common

Distant cousins : second cousin ;the children of first cousins (share the great grandparent) while third cousins: grandchildren of two first cousins (share the great great grandparent)[10]

Equipment:The HPLC system consisted of degaser YL 9101, the pump was YL 9110 quaternary pump, and the injection valve Rheodyne was equipped with 20 µl sample loading loop, detector model 1100 Agilent fluorescence. Collection and Processing of blood Sample .Venous blood was collected in heparin containing tubes. After centrifugation (2000 for 10 min). Add 20 µl from sulfosalicylic acid to deproteinize 200 µl of plasma; mixed on a vortex, incubate at 4C for 30 min, centrifuged at 3000g for 15 min remove precipitated protein. The clear supernatants were transferred to polypropylene vials and stored frozen at -20 C. Then derivatization step by adding to 30 µl of standard solution or plasma a 30 µl from Ortho-phthaladehyde (OPA) reagent and inject 20 µl onto the column.

Results : Out of 500 patients with neurological symptoms and /or sign who referred to quantitative amino acids by fluorescent HPLC, only 50 patients were detected to have significant Aminoacidopathies in the study, the overall analytic yield was 10%.

Demographic characteristics of study participants

Age of the patients at diagnosis

The mean age at diagnosis of AAs disorders (Table-1) shows that children diagnosed as HCU was the older with a mean of 64.6±57.3 months, next to it are children diagnosed as PKU 45.8±38.6months and child who was diagnosed as MSUD were the youngest 17.1±16.6 months.

Amino acid disorders were more common in male 62% and the male / female ratio for was 1.63. The male / female ratio for the HCU patients was 1.0, of the MSUD patients were 3.1, of the PKU patients were 1.4.

The Consanguinity among families of our patients shows about 82% of all AA disorders were 1st cousin, for details 86% of HCU patients, 78% of MSUD patients and 87% of PKU patients were 1st cousin (Table -1).

Table -1: Demographic characteristics of study participants.

		HCU	MSUD	PKU	Others	All
Age (mean±SD)		64.6± 57.3	17.1± 16.6	45.8± 38.6	21.3± 23.4	43.3± 44.4
Sex	Male	7	7	14	3	31
	Female	7	2	10	0	19
	M/F ratio	1.0	3.1	1.4	3.0	1.6
Consanguinity	1st cousin	12	7	21	1	41
	Distant cousin	1	2	3	0	6
	Not relative	1	0	0	2	3

Detection of Abnormal Amino Acids:

Two types of HCU were diagnosed, the classical HCU in which both homocysteine (HCY) and methionine were increased and the remthylation type of HCU in which HCY was increased with normal or low methionine.

Three types of MSUD were diagnosed; the classical type (increase valin, isoleucine and leucine), the intermittent type (increase valin, isoleucine and leucine) and the variant type (increase valin and leucine).

In all types of PKU there is increase PA and PA/ tyrosine ratio.Non-ketotic hyperglycinemia (NKH) increase glycine in plasma and CSF; also, Citrullinemia markedly increase citrullin and Tyrosinemia markedly increase tyrosine. (Table -2).

Table -2: Abnormal Amino Acids detected in patients.

AA disorder	type	Abnormal AA			n	%
HCU	(classical type)	increase methionine	+	increase HCY	7	14
	(remethylation type)	normal methionine	+	increase HCY	6	12
		low methionine	+	increase HCY	1	2
MSUD	(classical type)	increase valine & isoleucine	+	increase leucine	7	14
	(variant type)	increase valine	+	increase leucine	1	2
	(intermittent type)	increase valine & isoleucine	+	increase leucine	1	2
PKU	(classical and variant type)	increase PA	+	increase PA/tyrosine ratio	24	48
Other	NKH	increase glycine (plasma & CSF)	+	tyrosine	1	2
	Citrullinemea	increase citrulline	+	----------	1	2
	Tyrosinemea	marked increase tyrosine	+	----------	1	2
Total of all patients					50	100

Clinical Presentation of AA disorder patients:

Overall, participants shows that psychomotor delay represents the most common presenting sign (68%), among the HCU patients (72%), the MSUD patients (78%) and the PKUpatients (67%). The other clinical presentation forms are autistic spectrum disorder (8%) followed by spastic cerebral palsy among the PKU patients, hemiplegia (6%) among the HCU patients and acute encephalopathy (4%) among other AA disorder patients (table -3).

Discussion: In recent years, there have been considerable advances in the development of diagnostic test of IEM, such as tandem mass spectrometry (MS/MS), however, analysis of AAs in the physiological fluids by HPLC is still an indispensable diagnostic work-up. [11]

This is the first comprehensive study highlighting the clinical and biochemical diagnostic approaches to AA disorders in Iraq. Although neonatal screening by

MS/MS may enhance early detection, HPLC remains the key diagnostic tool for confirmation of all suspected cases, whether clinically symptomatic or initially positive by neonatal screening program. However, in a developing country like Iraq, resources are carefully allocated and testing is targeted based on clinical awareness.

Table -3: Clinical Presentation of AA disorders in all patients

Clinical Presentation of Diseases					
	HCU n %	MSUD n %	PKU n %	Other AA n %	All patients n %
Abnormal screening	1 2	1 2	1 2		3 6
Psychomotor delay	10 20	7 14	16 34	1 2	34 68
Hemiplegia	3 6				3 6
Disturbed consciousness (Acute encephalopathy)		1 2		2 4	3 6
Autistic spectrum disorder			4 8		4 8
Spastic cerebral palsy			2 4		2 4
Learning disability			1 2		1 2

Table -4: A disorders detected in selective screening of IEM in distinct population

AA disorder	PKU	MSUD	HCU
Iraq present study (10 mo.) 2014	24(48%)	9(18%)	14(28%)
San Deigo, USA1984 (3yr.) [22]	15(33.3%)	7(15.6%)	3(6.7%)
Germany (17yr.) 1994 [23]	24(20.2%)	14(11.8%)	1(0.8%)
Kuwait (3yr) 2011 [12]	10(37%)	1(3.7%)	5(18.5%)
Thailand (8yr.) 2012 [11]	13(22.4%)	20(34.5%)	2(3.5%)
Lebanon (12yr) 2013 [4]	90(42.7)	40(19)	8(3.8)

The overall analytical yield of 10% for AAs disorder detected by HPLC in present study was higher than that reported for other developing countries in the region such as Lebanon (8.8%),[4] Kuwait(4.4%)[12] and reported yields for AAs disorder detected by HPLC from other countries such as Thailand (5%)[11]. In present

study, there was considerable delay in diagnoses with mean age 36/12 years and only 3 patients (one for each PKU, HCU and MSUD) were screened early because of previous same condition in family. Therefore, identification and diagnosis of such disorder remain a real challenge and this agree with study done in other developing countries like Lebanon [4],whereas it is inconstant with study done in Thailand [11],this discrepancy related to establishment of neonatal screening ,which had been established in Thailand ,but it does not in our country, nevertheless, PKU now days can be detected in the neonatal period by neonatal screening program at presymptomatic or asymptomatic stage ,which may allow in some cases early therapeutic intervention and better outcome.[13]

However most of PKU in present study born before the newborn screening program implemented or they are from areas not yet involved by screening and regarding other AAs disorders already not involved by screening and their diagnosis depend on high index of clinical suspicion because early detection could lead to favorable outcome unfortunately we are still at the beginning of neonatal screening program, while routine screening of all newborns for inherited disorders began in the 1960s after Horst Bickel had established an effective dietary therapy for PKU, and Robert Guthrie introduced a simple bacterial inhibition assay to defect elevated concentrations of phenylalanine in dried blood spots. [1]

Fourteen patient (28%) of the total cases in the study that diagnosed with HCU as there is marked increased plasma HCY level,50% with marked elevation of methionine and that is classical HCU while other half there is marked increased in HCY with normal or low methionine that suggest remthylation type. In comparison with study done in Lebanon 8(3.8%) [4] and study done in Thailand 2(3.4%), [11] in these studies the type of HCU were classical (i.e. increase HCY and methionine) while in this study 7(14%) were classical and 7(14%) were remthylation type and the cause of increased our percentage caused by lacking screening program for HCU and the present of half of cases of HCU due to remthylation may be related to genetic factor or due short period of the study, but this study coincide with Lebanon[4] and Thailand[11] studies in late onset of diagnosis of HCU. In this study the psychomotor delay is the main clinical presentation at time of diagnosis of HCU and constitute 72% of cases and 21% experience thromboembolic phenomenon while only 1(7%) come for screening in early infancy

because he has had previous same disease in the family, although in the study there is 50% of cases with HCU they have positive family history. Clinical presentation and outcome of psychomotor delay and intellectual disability are present only in 50% of cases in study done in Lebanon [4] and 2(100%) of cases in study done in Thailand but of mild degree according to their outcome[11] and this discrepancy may be related to dietary regimen or genetic factors. About 43% of HCU experience frequent seizure most of them GTCS, although one case display classical epileptic spasm and it is not present such finding in studies done in Lebanon and Thailand.[4][11]

All patients with HCU sent for ophthalmological assessment and most of them prescribed to them glasses and labeled as refractory error and only one case has typical ectopia lenti and therefore there is demand for further assessment as there is possibility of underestimation of ectopia lenti.

Marfan habitus present in 35% & those who usually started to have such skeletal feature in late childhood & this consist with other studied done in Lebanon & Thailand. [4, 11]

In preset study, prospectively diagnosed 24 cases with PKU; the disease was more common in boys than girls (58% versus 42%). The mean age of the studied patient was 38/12 year and this correlate with study done in Tunisia by (Khemir et al) [14] and in Egypt by (A.A. Sadek et al). [15]

In our series, all patients with PKU have been markedly increasing PA and PA/tyrosine ratio detected in plasma samples by fluorescent HPLC.

The dominant clinical presentation of PKU was psychomotor delay 66%, ASD 16% and these coincide with study done in Egypt by A.A. Sadek et al (66.7, 33.3 respectively).[15]

The precise etiology of psychomotor delay and the mental retardation in untreated PKU is not understood. Because, excessive PA inhibit cerebral protein synthesis. It is possible that defective brain myelination may be related to deceased biosynthesis of myelin proteins [16]. The seizure is not prominent in our PKU cases while it is dominant in studies done by Khemir et al & Karim Zadeh. [14, 17], these disagreement in finding in present study with Khemir et al and Karim Zadeh, might be related to different type of presentation of PKU variants ,compared with classical PKU ,BH4 deficiencies are more sever and BH4 is not only a cofactor for PAH, but also for enzymes within the biosynthetic pathways for important neurotransmitter and it is associated with multiple sever neurological abnormalities. [18]

In patient with MSUD, it is important to evaluate the patient's physical, biochemical and clinical status as our patient diagnosed by HPLC as 7(78%) classical type with increase leucine, isoleucine & valin & intermittent type 1(11%) in which same AAs increment but occur only in stressful condition in addition to 1(11%) other disorders involving branched chain AA; Hyperleucine- isoleucinaemia & hypervalinaemia (MSUD variant). [7]

The psychomotor delay is the main presenting symptom in MUSD in about 78% and disturbed consciousness in 1(11%) who diagnosed as intermittent MSUD & this in contrast with Thailand study where

diagnosed at early infancy (1st 5 mo.) period during work up for neonatal encephalopathy work up [11], and it might be attributed to attended patients in present study who survive the initial metabolic crisis typically have neurodevelopmental delay and learning deficit. [9]

Two third of MSUD patients experienced GTCS while the seizure (GTCS) present in only ¼ of patients in Lebanon study [4],this difference might be related to dietory regimen adapted by different societies.

Although the history of maple syrup urine odor has inquired only in 3 patient but when repeat the question or smell the urine, we find it is present in most of cases and this is because some of parents, they don't experienced maple syrup odour. There for the smell of patient urine may be considered as part of clinical assessment in suspected patient with metabolic diseases.

Consanguinity was found in form of 1st cousin in 87% of cases of PKU, 86% of HCU and 78% of MSUD. And all cases 1st cousin consanguinity represent 80% and it is higher than study done in Lebanon [4] and as long as majority of metabolic diseases are autosomal recessive inherited traits occurring frequently in countries with high consanguinity rates, [19] which may suggest possibility of high rate of occurrence of IEM in Iraq although comprehensive published report are not available

PKU was the most prevalent AAs disorder in this study & this is coincided with the other IEM studies from several countries (table-4). In which PKU are the most prevalent disorders nevertheless, MSUD was still the most prevalent AA disorder in Thailand and Filipino, [11, 20] could be explained by founder mutation in the dihydrolipoyl transcyclase (E2). [21]

Psychomotor delay is the most prominent presenting sign in amino acid disorders in present study (68%) and it correlate with Karim et al study(50%) [16],therefore proper assessment psychomotor delay for potential AA disorder ,which is might be improved by dietary regimen and this need clinical awareness and accurate biochemical analysis as key role for diagnosis particularly in countries like Iraq with lacking comprehensive neonatal screening and aminoacidopathies cases are still diagnosed clinically.

Limitations: In this study, we could diagnose 50 cases with AA disorders during 10 months; a period which considered remarkably short in comparison with other studies from several countries. (Table -4) Unfortunately the diagnosis was delayed due to shortage of facilities and lack of expanded neonatal screening program.

Conclusions:

1. Several Amino acid disorders in high-risk patient could be diagnosed by HPLC.
2. Consanguinity had a definite role in the increased frequency of metabolic disease in our population as most of them are autosomal recessive Mendelian inheritance.
3. The tragedy, of late diagnosis, of Amino acid disorders was still considered challenging issue.
4. Different clinical presentation and the intersection between Amino acid disorders required high index of suspicion, particularly in our community with a high rate of consanguinity and inbreeding.

Recommendations:

1. Expanding and extending of tandem mass LC-MS/MS to involve all Iraq with involvement other AA disorder like HCU & MSUD.
2. Establishment of specific metabolic centers in various universities and research institutes and provided them with advanced metabolic diagnostic equipment.
3. Establishment of Health education program about early detection of metabolic disorder in term of psychomotor delay, urine and sweat odor, positive family history of AA disorders and education about the consanguinity and its role in metabolic disease

List of Abbreviations:

AA	Amino acid
ASD	Autistic spectrum disorder
CBS	Cystathionine-β-synthase
HPLC	High performance liquid chromatography
HCY	Homocysteine
HCU	Homocysteinurea
IEM	Inborn error of metabolism
MSUD	Maple syrup urine disease
NKH	Non-ketotic hyperglycinemia
Phe	Phenylalanine
PKU	Phenylketonuria
PAH	Phenylalanine hydroxylase
Tyr	Tyrosine
UCD	Urea cycle disorder

Competing interests;the authers declare that they have no competing interest

Authors contributions :A. K. did the literature research, analysed the data and drafted the manuscript. N. A. and H.H. were involved in discussion and evaluation of the data and critically revised the manuscript and also participated in the study coordination and helped to draft

the manuscript in addition to contributed clinical data. All authors read and approved the final manuscript.

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