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Concentration of the Main Biochemical Blood Indices in Early Postnatal Period

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Abstract

The work deals with the analysis of the umbilical blood samples (120 samples) concerning the concentration of general protein and glucose as the main biological constants of newborns. There is no a reliable difference found between these indices while comparing groups with monocyesis and twin pregnancies. Possible correlation interrelation of certain biochemical indices in newborns during early postnatal period was examined (54 samples). In addition, changes of the examined biochemical indices were followed in the course of time in 19 newborns concerning the comparison of umbilical and venous blood.

Keywords: umbilical blood of monocyesis and twin pregnancy, blood of newborns, glucose, general protein, urea, creatinine, bilirubin, correlation interrelation.

Introduction

Detection of the levels of glucose and general protein concentration in the umbilical blood of newborns is one of the most important homeostatic parameters which is of great value for postnatal adaptation. As a rule, detection of these common biochemical parameters is indicated for the newborns from “risk group”: preterm babies with low body weight, intrauterine hypotrophy, born to mothers suffering from diabetes mellitus or obesity, etc.

According to literary data risk factors of the intrauterine growth and development of the fetus are pre-eclampsy on late terms of gestation, diabetes mellitus, viral infection (usually cytomegalovirus), heart defects with circulatory disorders, which evidently results in disorders of homeostasis including decrease of glucose and general protein levels [1-3].

Literary evidence concerning the norm of protein concentration in term and preterm newborns in the blood serum range approximately within the following values: 46,0 – 68,0 g/L and 36,0-60,0 g/L respectively [1].

As to the question of concordance of glucose standard levels in the blood of newborns, diametrically opposite views are found in scientific literature concerning hypoglycemic importance

of sugar level in the blood, not only depending on gestational age of a newborn and body mass index, type of feeding, region of the study and even daily period of a baby's life activity [3, 4, 6].

In 2000 a group of expert headed by Cornblath M., came to the conclusions that hypoglycemia can be found as a pathologic reaction to glucose insufficiency for the CNS first of all. The safe glucose level in early neonatal period despite the term of gestation is 2,5 mmol/L. These data correspond to the criterion of hypoglycemia according to Shabalov N.P. (2004) – less than 2,6 mmol/L in any time of a day [3]. The study of Anderson D.M.et al. is indicative of hypoglycemia found in 38% of newborns under condition of critical threshold value of 2,6 mmol/L in the first 50 hours of life [3, 5, 8].

It should be noted that the most frequent disorders of glucose metabolism are found in sick newborns, especially preterm ones, causing their susceptibility bot to hypoglycemia and hyperglycemia [1].

Duration of labour period, surgical delivery and even a kind of anaesthesia are able diametrically, in a short period of time, to change the dynamics of glycemia of a newborn [1, 9].

All these factors are topical for Ukraine as well, where the share of preterm labour achieves 5% from general number. At the same time, 10-12% of newborns in Ukraine have their body mass lower than 2500,0 g. Sickness rate in this category of infants is three times higher than that of the category with body weight higher that threshold value of 2,5 kg. These are the infants with insufficient body mass that are characterized by a high risk of disorders of postnatal adaptation processes and following possibility to develop numerous pathological conditions, which stipulates the necessity to correct the processes of life activity including metabolic disorders [7].

Approximately one third of newborns with the body weight less than 2,5 kg is infants with retardation of intrauterine growth and development. With the aim to unify the requirements and capacity to the quality of medical care of newborns with low body mass the Order of the Ministry of Public Health of Ukraine № 584 dated 29.08.2006 «On the Approval of the Protocol of Medical Care of a Newborn with a Low Body Mass at Birth» was introduced, where the critical glucose level ranges within 2,2 – 2,6 mmol/L [7, 8].

A normal glucose level for newborns according to this Protocol is within the ranges of 2,6 to 5,5 mmol/L. Glucose level in the blood of newborns is recommended to be measured by means of gluco-test, in case the result of 2,6 mmol/L and lower is obtained, laboratory detection is recommended with the use of unified methods to prevent physiological conditions of hypoglycemia [7].

Not the least of the factors is that glucose level in the blood plasma is by 18% higher than in the whole blood, in addition, hyperbilirubinemia and hemolysis result in false reduction of glucose concentration with the use of test-strips and reliability of results become lower to 75-85% [3].

Materials and methods

During the period from April 2014 to June 2015 on the base of biochemical laboratory at the Department of Anaesthesiology with intensive care units of the Municipal Clinical Maternity Home №1, the town of Chernovtsy (Ukraine), 120 samples of umbilical blood of newborns were examined to detect the concentration of general protein and glucose according to the Protocols of management of newborns. Among those samples there were blood from 14 pairs from the category of twins (n = 28). For further correction of metabolic processes and dynamics of treatment of newborns 54 samples of venous blood of infants were examined. It should be noted that in 15 individuals from this number the examinations of the concentration of general protein and glucose were made at least twice: in the umbilical blood and then – in the blood of a newborn. In four cases metabolic changes of the concentration of general protein and glucose were made only in the venous blood serum as at the moment of birth there were no necessity to conduct these biochemical examinations.

Moreover, in the blood serum of newborns and in two samples of umbilical blood the concentrations of urea (n = 34) and creatinine (n = 27) were examined.

Umbilical blood was taken by obstetricians in the labour ward immediately in the first minutes after birth of a baby. Venous blood was taken by the medical staff in the Department of intensive care of newborns.

For further examination blood was delivered as soon as possible to the laboratory, centrifuged at 1500-2000 rotations per minute to get serum.

The concentration of metabolites in the blood serum was detected by means of unified common methods with the use of reagent sets of the firm "Reagent", Dnepropetrovsk, Ukraine: protein concentration – by biuretic method, glucose – by enzymatic glucose oxidase one, urea – by enzymatic urease one, bilirubin – by Jendrassik method, creatinine – by kinetic method without deproteinization (Jaffe reaction).

To measure optic density of solutions the photocolorimeter «Solar» PM-1111was used.

Statistical processing was conducted by means of detection of the mean value of indices and their average quadratic deviation with the use of different groups of methods of non-parametric analysis to compare them: the criteria of Spearman, U-Whilkokson-Mann-Whitney, T-Whilkokson. Spearman's rank correlation coefficient was used to detect and estimate the closeness of relations between the number of comparing quantitative indices in the umbilical blood.

T-Whilkokson criterion was used to compare the indices measured in two different conditions on the same sample of the examined objects (umbilical and venous blood of infants).

Results and discussion

Level of general protein in the umbilical blood was within the range from 37,8 g/L to 69,4 g/L. An average amount of general protein concentration in the umbilical blood was 49,6 g/L.

It was an interesting fact that there was no reliable difference in comparison of the indices of general protein and glucose concentration between monocyesis and twin pregnancy by U-Whilkokson-Mann-Whitney criterion (table 1). Vitally important indices were in equal limits of ranging irrespective of a number of developing fetuses.

As to glucose level in the umbilical blood, its concentration was from 2,2 to 5,9 mmol/L. Seven results out from 120 characterized glucose concentration equal or lower than 2,6 mmol/L, but not lower than 2,2 mmol/L (2,2 ≤ glucose ≥2,6). Three from these hypoglycemic indices belonged to twins.

Table 1: Concentration of certain biochemical indices in the umbilical blood of monocyesis and twin pregnancy

		General protein (g/L)	Glucose (mmol/L)	General bilirubin (mkmol/L)
Monocyesis (n=92)	M ± m	49,60 ± 0,78	4,20 ± 0,12	40,39 ± 2,21 (n=40)*
Twins (n=28)	M ± m	49,60 ± 0,93	3,73 ± 0,18	36,53 ± 3,29 (n=12)*

* – amount of concentration detection of general bilirubin level in the serum of umbilical blood.

As Table 1 shows the amount of concentration detection of general bilirubin level is substantially lower than the indices of general protein and glucose. Bilirubin in this series of examinations was secondary index and was indicative of a possible group or rhesus conflict in "mother-baby" system against the ground of primary problem of immaturity of a newborn organism or any other disorders of postnatal adaptation.

It is interesting that one index of glucose in the umbilical blood was 9,2 mmol/L. The mother of this infant was afflicted with type I diabetes in anamnesis, obesity (body weight more than 120 kg), hypertension, and as a result, pre-eclampsy on late terms of gestation. The woman stayed in the hospital for about two months in the department of pathology of pregnancy and anaesthesiology with beds for intensive care therapy (delivery by means of cesarean section).

Literary sources are indicative of a reverse relation between sugar level in the blood of newborns and degree of obesity in mothers. In case of carbohydrate metabolism pathology the frequency of hypoglycemia achieves 38,5 ± 14,0 % [4]. In addition, in infants born to mothers with insulin-dependent diabetes mellitus or experienced diabetes of pregnancy, transitory hypoglycemia develops.

Our case of excluded from rules, and we could not but mentioned it, but further detection of glucose in this infant by glucose-oxidase laboratory method was not performed.

According to the statistics we have not found the interrelations between the indices of general protein and glucose in the umbilical blood both in monocyesis and twin pregnancies. On the basis of scientific literary data concerning this question, in infants with much lower glucose level in the

umbilical blood the lower indices of general protein were registered more frequently statistically [2]. It should be noted that our study included only 120 blood samples as compared with 331 samples described in the study performed by Karpov F.L. and Miroshnichenko O.A. In addition, the group of 331 newborns included only healthy term infants. Our criterion of the study was the fact to detect these indices in the blood of newborns irrespective of their gestational age and baby's condition (prescribed by a neonatologist). The authors of the investigation indicate to the interrelations of these important biochemical indices from the view of supplying energy balance in the organism of a newborn [2, 10].

Examination of infants in their first hours of life demonstrated how fulminant (in the first hours of the first day) and characteristically pronounced the changes in the blood system were. Duration of labour stress, oxygen insufficiency influence upon the dynamics of blood indices greatly [9].

To characterize metabolism and control the treatment in newborns, in addition to the indices of glucose, general protein and bilirubin, the concentrations of urea and creatinine were detected as required. While comparing these indices by Spearman's correlation criterion we have got the results presented in Table 2.

According to these data, there was an average connection between the levels of general bilirubin and urea (r = 0,57), as well as between bilirubin and creatinine (r =0,57); a moderate connection between urea and creatinine (r = 0,40) in the venous blood of newborns found.

In healthy infants physiological jaundice may occur against the ground of immature liver (enzymatic systems). Increased bilirubin level in the blood of preterm newborns does not depend on the body mass at birth, but it correlates directly with the stage of fetus maturation and maternal diseases during pregnancy. The correlation between pigment and nitrous metabolism in newborns is indicative of the formation of important functional systems of the liver involving in the regulation of the main metabolic ways.

Table 2: Correlation of the venous blood indices in newborns in early postnatal period by Spearman's correlation criterion

	General protein (g/L) (n = 54)	Glucose (mmol/L) (n = 41)	General bilirubin (mcmol/L) (n = 19)	Urea (mmol/L) (n = 34)	Creatinine (mcmol/L) (n = 27)
General protein (g/L)	---	-0,004	0,1063	-0,215	-0,053
Glucose (mmol/L)	---	---	0,0629	0,1323	0,1936
General bilirubin (mcmol/L)	---	---	---	0,5714*	0,5714*
Urea (mmol/L)	---	---	---	---	0,4042*
Creatinine (mcmol/L)	---	---	---	---	---

Note: * – reliable correlation between the indices ($p < 0,05$).

Nitrous metabolism in children differ by a number of peculiarities, and a positive nitrous balance as an essential condition of growth in particular. The intensity of nitrous metabolism processes during the infant's growth undergoes considerable changes: during the first three days of life nitrous balance is negative, which is explained by insufficient intake of protein with food. In this period transient increase of residual nitrogen concentration in the blood is found. Physiological creatinuria is characteristic feature. Creatine is found even in amniotic fluid; it is found in urine in the amounts higher than its content in the urine of the adults beginning from the neonatal period to the age of puberty. In the first days of life newborns may display transient peculiarities of metabolism characterized by proteinolysis (hypoproteinemia) together with hypoglycemia. In our case the level of urea concentration about 7,0 mmol/L is the characteristics of transient conversion from the intrauterine to neonatal period of life.

Unfortunately, according to the protocols of management of newborns and requirements of neonatologists we have not conducted the study concerning the concentration of urea and creatinine in the umbilical blood.

Analysis of literary data is indicative of the fact that the content of biochemical indices in the umbilical blood of newborns from the control group ranges within the standard indices of various sources, except the level of urea which was by 30% (5,2 mmol/L) lower than that of an average-standard one. It proves the necessity to work out personal standard indices for every laboratory considering the peculiarities of the cohort, region and other environmental conditions [11].

We had the opportunity to compare the main biochemical indices in the umbilical blood and blood of newborns in early postnatal period between themselves in 19 individuals. In addition to the examination of the umbilical blood at the moment of birth, the main biochemical constants in the venous blood were detected in those infants one time. It was made in 11 individuals and eight times twice (five cases) or even three times (three cases) depending on the severity of an infant's condition or the necessity to correct metabolism.

Table 3 presents the results of examination of correlative changes in biochemical indices of infants depending on the period of their life.

According to T-Whilkokson's criterion there are reliable changes between the concentration of protein in the umbilical blood and in the venous blood of an infant during the first blood sampling ($p < 0,05$). But during further detection of general protein levels in one and the same infant: blood №1 and №2, reliable changes between them were nor found. As to glucose reliable changes between the umbilical blood and venous blood were not found as well as in different samples of the venous blood in early postnatal period of infants' life.

We did not take into account two indices of the concentration of urea and creatinine in the umbilical blood serum as single parameters.

Table 3: Comparison of the dynamics of main biochemical indices in newborns in the process of their vital activity (M ± m)

	General protein (g/L)	Glucose (mmol/L)	General bilirubin (mcmol/L)	Urea (mmol/L)	Creatinine (mcmol/L)
Umbilical blood	48,15 ± 2,22	4,14 ± 0,24	33,8 ± 3,04	---	---
Infant's blood (№1)	50,19 ± 1,89	4,76 ± 0,42	159,82 ± 33,70	7,00 ± 1,06	108,93 ± 42,92
Infant's blood (№2)	49,53 ± 1,70	3,40 ± 0,33	130,53 ± 33,83	7,11 ± 1,49	144,20 ± 83,11

Note: blood № 1 and №2 – in one and the same individual in different periods of time.

There were no sufficient data to compare the indices of urea and creatinine.

It stipulates certain interest to further accumulation of a number of studies which will include no less than 300 samples of the umbilical blood and 150 samples of the venous blood for analytical and correlation analysis of biochemical constants including the activity of aminotransferase enzymes (ALT, AST) as important indices in liver metabolism (transamination reaction) and inclusion of protein products (amino acids) in the process of gluconeogenesis.

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Концентрация основных биохимических показателей крови в раннем постнатальном периоде

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Аннотация. В работе проведен анализ образцов пуповинной крови (120 образцов) относительно концентрации общего белка и глюкозы, как основных биохимических констант новорожденных. Показано, что нет достоверной разницы между этими показателями при сравнении в группах одноплодных и двойни. Исследована возможная корреляционная взаимосвязь некоторых биохимических показателей у новорожденных в раннем постнатальном периоде (54 образца). Кроме того, рассмотрены изменения исследованных биохимических показателей во временном периоде у 19 новорожденных при сравнении пуповинной и венозной крови.

Ключевые слова: пуповинная кровь одноплодных и двойни, кровь новорожденных, глюкоза, общий белок, мочевина, креатинин, билирубин, корреляционная взаимосвязь.

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Studying the Antimicrobial and Antiviral Effects of Electrochemically Activated Nacl Solutions of Anolyte and Catholyte on a Strain of *E. Coli DH5* and Classical Swine Fever (CSF) Virus

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Abstract

This paper outlines the results on the antiviral and antimicrobial action of electrochemically activated NaCl solutions (anolyte/catholyte), produced in the anode and cathode chamber of the electrolitic cell, on classical swine fever (CSF) virus and a stain of *E. coli DH5*. It was found that the anolyte did not affect the growth of the cell culture PK-15; the viral growth during the infection of a

cell monolayer with a cell culture virus was affected in the greatest degree by the anolyte in 1:1 dilution and less in other dilutions; whereas the viral growth at the infection of a cell suspension with the CSF virus was affected by the anolyte in dilution 1:1 in the greatest degree, and less by other dilutions; viral growth at the infection with a virus in suspension of the cell monolayer was affected by the anolyte in all dilutions. Unexpectedly, the stronger biocidal effect of the catholyte was observed when a strain of *E. coli DH5* was treated by the anolyte and catholyte, respectively. In order to provide additional data about the antiviral activity of the electrochemically activated water and the distribution of H_2O molecules according to the energies of hydrogen bonds, the non-equilibrium energy spectrum (NES) and differential non-equilibrium energy spectrum (DNES) of the anolyte and catholyte were measured.

Keywords: anolyte, catholyte, *E. coli DH5*, CSF virus, NES, DNES.

Introduction

The phenomenon of electrochemical activation of water (EAW) is a set of electrochemical and electrical processes occur in water in the electric double layer (EDL) type of electrodes (anode and cathode) with non-equilibrium electric charge transfer through EDL by electrons under the intensive dispersion in water the gaseous products of electrochemical reactions [1]. In 1985 EAW was officially recognized as a new class of physical and chemical phenomena.

As a result of the treatment of water by a constant electric current at electric potentials equal to or greater than the decomposition potential of water (1,25 V), water goes into a metastable state, accompanied by electrochemical processes and characterized by the abnormal activity levels of electrons, the redox potential, and other physical-chemical parameters (pH, E_h, ORP) [2].

The main stage of electrochemical treatment of water is the electrolysis of water or aqueous solutions with low mineralization as aqueous solutions of 0,5–1,0 % sodium chloride (NaCl) [3], which occurs in the electrolysis cell, consisting of the cathode and the anode separated by a special semipermeable membrane (diaphragm) which separates water to alkaline fraction – the catholyte and acidic fraction – the anolyte (Figure 1). When the passing of the electric current through water the flow of electrons from cathode as well as the removal of electrons from water at the anode, is accompanied by series of redox reactions on the surface of the cathode and the anode [4]. As the result, new elements are being formed, the system of intermolecular interactions, as well as the composition of water and the water structure are changed [5, 6].

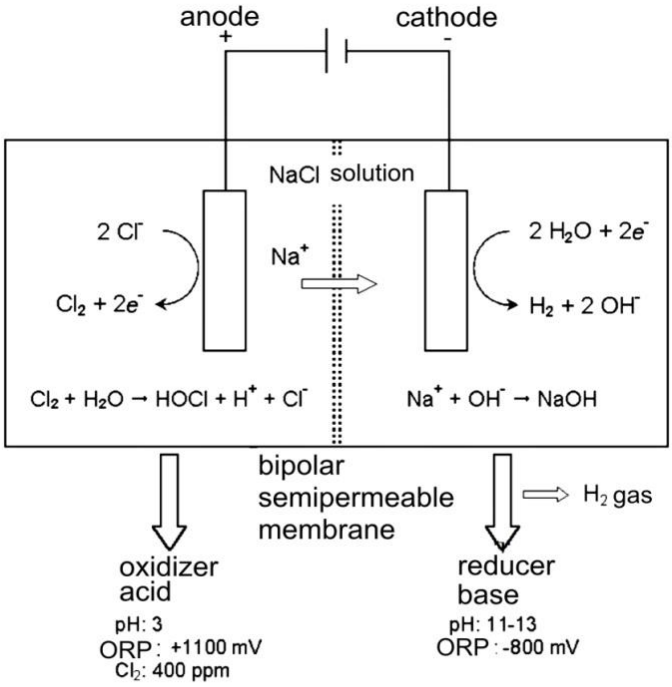

Figure 1. The diaphragm electrolysis method for the preparation of acid (anolyte) and alkali (catholyte) solutions via the electrochemical activation of sodium chloride

The products of electrode reactions are the neutralized aqueous admixtures, gaseous hydrogen and oxygen generated during the electrolytic destruction of H_2O molecules, metal cations (Al^{3+}, Fe^{2+}, Fe^{3+}) in the case of metal anodes made of aluminum and steel, the molecular chlorine. Wherein at the cathode is generated the gaseous hydrogen, and at the anode – oxygen. Water also containes a certain amount of hydronium ions (H_3O^+) depolarizing at the cathode with formation of the atomic hydrogen:

$$H_3O^+ + e^- \rightarrow H + H_2O, \quad (1)$$

In an alkaline environment there occurs the disruption of H_2O molecules, accompanied by formation of the atomic hydrogen and hydroxide ion (OH^-):

$$H_2O + e^- \rightarrow H + OH^-, \quad (2)$$

The reactive hydrogen atoms are adsorbed on the surfaces of the cathode, and after recombination are formed the molecular hydrogen H_2, released in the gaseous form:

$$H + H \rightarrow H_2, \quad (3)$$

At the same time at the anode is released the atomic oxygen. In an acidic environment, this process is accompanied by the destruction of H_2O molecules:

$$2H_2O - 4e^- \rightarrow O_2 + 4H^+, \quad (4)$$

In an alkaline environment, the source of oxygen source is OH^- ions, moving under the electrophoresis from the cathode to the anode:

$$4OH^- \rightarrow O_2 + 2H_2O + 4e^-, \quad (5)$$

The normal redox potentials of these reactions compiles +1,23 V and +0,403 V, respectively, but the process takes place in certain conditions of electric overload.

The cathodes are made of metals that require high electrical voltage (lead, cadmium), allow to generate the reactive free radicals as Cl*, O*, OH*, HO_2*, which react chemically with other radicals and ions.

In bulk oxidative processes a special role plays products of electrolysis of water – oxygen (O_2), hydrogen peroxide (H_2O) and hydrochlorine acid (HClO). During the electrolysis, an extremely reactive compound formed – H_2O_2, the formation of which occurs due to the hydroxyl radicals (OH*), which are the products of the discharge of hydroxyl ions (OH^-) at the anode:

$$2OH^- \rightarrow 2OH^* \rightarrow H_2O_2 + 2e^-, \quad (6)$$

where OH^* – the hydroxyl radical.

The chlorine-anion is transformed to Cl_2:

$$2Cl^- \rightarrow Cl_2 + 2e^-, \quad (7)$$

Gaseous Cl_2 forms highly active oxidants: Cl_2O; ClO_2; ClO^-; HClO; Cl^*; HO_2^*. The parameters of pH, the redox potential, ORP and the electrical conductivity of the anolyte/catholyte depend on different factors including the ratio of water volumes in the two electric chambers, the material of electrodes, NaCl concentration, the temperature, electric voltage and processing time [7,8].

The electrolysis cell can be regarded as a generator of the above mentioned products, some of them, entering into the chemical interaction with each other and water impurities in the interelectrode space, providing additional chemical treatment of water (electrophoresis, electroflotation, electrocoagulation) [9]. These secondary processes do not occur on the electrode surface, but in the bulk water. Therefore, in contrast to the electrode processes they are indicated as the volume processes. They generally are initiated with increasing the temperature of water during the electrolysis process and with increasing the pH value.

As a result of the cathode (catholyte) treatment water becomes alkaline: its ORP decreases, the surface tension is reduced, decreasing the amount of dissolved oxygen in water, increases the concentration of hydrogen, hydroxyl ions (OH^-), decreases the conductivity of water, changes the structure of hydration shells of ions [10]. By external characteristics the catholyte – is a soft, light, with an alkaline taste liquid, sometimes with white sediment; its pH = 10–11, ORP = -200...-800 mV.

On physical and chemical parameters the catholyte has the significantly enhanced electron-donating properties, and getting into the physiological fluids of an organism can enhance the electron-background for a few tens of millivolts [11]. The catholyte reportedly has antioxidant, immunostimulating, detoxifying properties, normalizing ORP, metabolic processes (increases the ATP synthesis, modification of enzyme activity), stimulates the regeneration of tissues, increases the DNA synthesis and stimulates the growth and division of cells by increasing the mass transfer of ions and molecules across the cell membrane, improves trophic processes in tissues and blood circulation [12]. It was also reported that catholyte with the redox potential at -700...-100 mV

favorizes the development of anaerobs, whereas the anolyte with the redox potential at +200…+750 mV supports the growth of aerobs [13]. The antibacterial effect of the catholite is differentiated: the bactericidal effect is appeared relative to Enterobacteriaceae, resistant to it are enterococci and the group of streptococci B, and against Gram-negative microorganisms – only the bacteriostatic effect [14].

The electrochemically activated solutions of the catholite, depending on the strength of the transmitted electric current may be of several types:

C – alkaline catholyte (pH > 9,0; ORP = -700…-820 mV), the active components – NaOH, O_2, HO_2^-, HO_2*, OH^-, OH*, HO_2^-, O_2;

CN – neutral catholyte (pH = 9,0; ORP = -300…-500 mV), the active components – O_2, HO_2^-, HO_2*, H_2O_2, H^+, OH^-.

As a result of the anode (anolyte) treatment water becomes acid reaction, the ORP increases slightly, the surface tension is slightly reduced, the conductivity increases, the amount of the dissolved oxygen and chlorine in water also increases, whereas the amount of hydrogen decreases [15]. The anolyte is a brownish, acid, with a characteristic odor and taste the liquid with a pH = 4–5 and ORP = +500…+1100 mV. The specific anolyte toxicity when being administered in the stomach and applying to the skin refers to the class 4 of harmful substances according to the Russian Standard GOST 12.1.007-76, with the minimal toxicity within this class. When being inhaled the anolyte with oxidants content of 0,02 % and total mineralization 0,25–0,35% does not irritate the respiratory system and mucous membranes of the eyes. When introduced into the organism, the anolyte has no immunotoxic action and increased chromosomal aberrations in the bone marrow cells and other tissues, and it has no cytogenetic activity. When being heated to t = +50 °C the bactericidal activity of the anolyte is increased by 30–100% [16].

The electrochemically activated solutions of the anolyte are divided into four main types:

A – acidic anolyte (pH < 5,0; ORP = +800…+1200 mV), the active components – HClO, Cl_2, HCl, HO_2*;

AN – neutral anolyte (pH = 6,0; ORP = +600…+900 mV), the active components – HClO, O_3, HO^-, HO_2*;

ANK – neutral anolyte (pH = 7,7; ORP = +250…+800 mV), the active components – HClO, ClO^-, HO_2^-, H_2O_2, O_2, Cl^-, HO*;

ANKD – neutral anolyte (pH = 7,3; ORP = +700…+1100 mV), the active components – HClO, $HClO_2$, ClO^-, ClO_2*, HO_2*, H_2O_2, O_2, O_3, Cl^-, HO^-, O*.

The anolyte has antibacterial, antiviral, antifungal, anti-allergic, anti-inflammatory, antiedematous and antipruritic effect, may be cytotoxic and antimetabolite action without harming the human tissue cells [17]. The biocide elements in the anolyte are not toxic to somatic cells, as represented by oxidants, such as those ones produced by the cells of higher organisms.

Studies on the virucidal effect of the anolyte are rare and insufficient, basically on the possibilities of applying the anolyte in the implementation of effective control of viral diseases in humans and animals and especially on particularly dangerous viral infections, as staphylococcal Enterotoxin-A [18]. One of them is the classical swine fever (CSF), prevalent in different regions of the world and inflicting heavy economic losses. It is caused by enveloped viruses belonging to the genus *Pestivirus* of the family *Flaviviridae* [19, 20]. The resistance and inactivation of the virus of CSF virus is a subject of extensive research. Although it is less resistant to external stresses other than non-enveloped viruses, it retains its virulence for a long period of time: in frozen meat and organs – from a few months up to one year; in salted meat – up to three years; in dried body fluids and excreta – from 7 to 20 days. In rotting organs it dies for a few days and in urine and faeces – for approx. 1–2 days. In liquid fertilizer it can withstand 2 weeks at t = +20 °C, and over 6 weeks at t = +4 °C. Its thermal resistance may vary depending on the strain type, but the inactivation is dependent mostly on the medium containing the virus. Although the CSF virus loses its infectivity in cell cultures at t = +60 °C for 10 min, it is able to withstand at least 30 min at t = +68 °C in defibrinated blood. It is relatively stable at pH = 5–10, and the dynamic of the inactivating process below pH = 5 depends on the temperature.

According to J.A. Sands [21] and U.S. Springthorpe [22], the effective disinfection of viruses whose infectivity is associated with the elements of the casing is achieved by disinfectants dissolving fats, surfactants, disinfectants or fatty acids, organic solvents (ether and chloroform), detergents, proteases, and common disinfectants. It is believed that 2% solution of sodium hydroxide is most

suitable for the disinfection of spaces contaminated with them. It is thought that to achieve the effective electrochemical disinfection it is necessary to irreversibly damage the RNA [23].

Investigations conducted by other authors [24] were carried out with *E. coli*, using as a desinfectant the anolyte with ORP equal or greater than +1100 mV and pH = 5,5, obtained via electrolysis of diluted NaCl solution on planktonic cells of a strain of *E. coli JM109*. It was demonstrated that within 5 min of influence all cells were inflated and burst. Also, it was occurred a full destruction of proteins, DNA and RNA. Supposedly the anolyte enters the cells provoking structural and functional damages on the cell's membrane and cell's wall.

Similar research was performed by S.V. Kumar et al. [25]. They evaluated the inactivation efficacy of anolyte of pH = 2,7 and ORP = + 1100 mV on *Escherihia coli O157:H7*, *Salmonela enteritidis* and *Lusteria monocytogenes*. As it was demonstrated on five strains of *E. coli E06* (milk), E08 (meat), E10 (meat), E16 (meat) and E22 (calf feces), all patogens were significantly reduced (7,0 logCFU/ml) or fully destroied (8,0 logCFU/ml) after 2 to 10 min inactivation by the anolyte in the temperature range from t = +4 °C to +23 °C. Supposedly, the low pH value of the anolyte makes sensitive the outer cell's membrane, thus facilitating HClO to enter the cell and further destroy it.

However, it should be noted that the pharmacological studies of electrochemically activated solutions of NaCl and their virucidal effects and toxicity have not yet been completely evaluated. Therefore, the purpose of this research was to study the antiviral virucidal effect: 1) of the anolyte in different dilutions on classical swine fever virus in cell culture and organ suspensions; 2) of the anolyte/catholyte on a strain of *E. coli DH5a*, and 3) to determine how the virocidal effect relates to local maximums in NES-spectra of the anolyte and catholyte*.

Material and Methods

Methods

The study of the antiviral activity of the anolyte were performed at the National Reference Laboratory of Classical and African Swine Fever, section "Exotic and Especially Dangerous Infections" of the National Diagnostic and Research Veterinary Medical Institute (Sofia, Bulgaria). The study on the antimicrobial activity of the anolyte/catholyte was performed at the Institute of Molecular Biology of the Bulgarian Academy of Sciences (BAS). Experiments were conducted with the anolyte obtained by the electrolysis apparatus "Wasserionisierer Hybrid PWI 2100" equipped with four titanium electrodes coated with platinum. 0,3% solution of chemically pure sodium chloride (NaCl) in distilled water was used for the electrolysis: the anolyte had ORP = +1070 mV and pH = 3,2; the catholyrte had ORP ≈ -180 mV and pH = 9,8.

Objects of study

Bacterial strain used in these experiments was *E. coli* DH5α with genotype: *fhuA2 lac(del)U169 phoA glnV44 Φ80' lacZ(del)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17.* A cell culture of porcine origin sensitive to the CSF virus was used: a continuous cell line was PK-15. Contamination of cell cultures was carried out with the standard cell culture test virus 2,3 (Bulgaria) with a cell titre 107,25 $TCID_{50}$/ml and organ suspension of internal organs (spleen, kidney, lymph node) of wild boar originating from the last outbreak of CSF in Bulgaria in 2009. The titer of the established virus in the suspension was $1 \cdot 10^{4,75}$ $TCID_{50}$/ml.

Studying the virucidal activity of the anolyte

To establish the virucidal activity of the anolyte, the inocula prepared for contamination of cell culture (cell culture virus) were treated at t = +22 °C with the following dilutions of the anolyte in sterile distilled water: 1:1 (50%), 1:2 (33%), 1:3 (25%), 1:4 (20%). These dilutions were mixed with inocula in proportion 1:1 (100 μl of the CSF virus suspension and 100 μl of the appropriate anolyte concentration). The time of action was conformed to the period, at which it was methodologically necessary to "capture" any viral presence in the cell culture. Upon the infection of a cell monolayer, the mixture was removed after the end of the exposure period of 1 h. Upon the infection of a cell suspension, the mixture, otherwise, was not removed.

* Such a dependence was established between the local maximum (-0,1387 eV; 8,95 μm) in the NES-spectrum of the catholyte that suppresses the development of tumor cells (Ignatov & Mosin, 2014).

Studying the virucidal activity of the anolyte

To establish the virucidal activity of the anolyte on the CSF virus in the suspension, a different dilution was used: the inoculum was mixed directly with the concentrated anolyte in anolyte-inoculum ratios 1:1; 3:1; 7:1 and 15:1 respectively. Since it is known that the growth of the virus does not cause a cytopathic effect, therefore, for demonstration of its presence, immunoperoxidase plates dyeing were used. The cells were fixed and the viral antigen was detected after binding to a specific antibody labeled with peroxidase. The organs exude 1 cm^3 of tissue, which was homogenized in a mortar with 9 ml of the cell culture medium containing antibiotics, in order to obtain 10 % of organ suspension. Sterile sand was added to improve the homogenization. The samples were kept at room temperature for 1 h, after that they were centrifuged for 15 min at 2500 g. The supernatant was used to infect the cells. In case of cytotoxic effect, the parallel dilutions of the homogenates were prepared in proportions 1:10 and 1:100. From the suspensions into multi well (24-well) plates were added 200 µl of the inoculums with coverage of 50–80%. Cell cultures were incubated at t = +37 °C for 1 h in order to "capture" an eventual virus if presented, then they were rinsed once with PBS and fresh media were added. Alternatively, the plate was filled directly (cell suspension), since the preliminary studies had found that the anolyte did not induce a cytotoxic effect.

The immunoperoxidase technique

The cell cultures were incubated for 72–96 h at t = +37 °C in a CO_2 incubator. The procedure with preparation of the positive and negative control samples was similar. The positive control sample was a reference strain of the CSF virus. The immunoperoxidase technique with using a horseradish peroxidase was used for the enzymatic detection of antigen-antibody complexes in cell cultures. The fixation of the plates was carried out thermally for ~3 h at t = 80 °C in a desiccator. In the processing was used a primary monoclonal antibody C 16, diluted in proportion 1:50, and secondary antibody RAMPO, diluted in proportion 1:50. For the immunoperoxidase staining was used 3 % H_2O_2 and AEC (dimethylformamide and 3-amino-9-ethylcarbazole) in acetate buffer. The antibody-antigen complex was visualized by the peroxidase reaction with the substrate.

Polymerase chain reaction (PCR)

The PCR to amplify the segments of the RNA was carried out in real time scale. The cell culture and organ suspensions were examined for the presence of the CSF viral genome by the PCR in real time (real-time RT-PCR, one step, TagMan), one-step according to Protocol of the Reference Laboratory for CSF of EU. For RNA extraction was used the test QIAamp Vital RNA Mini Kit, Qiagen Hilden (Germany). The initial volume of the biological material was 140 µl, and the elution volume – 60 µl. For amplification of PCR was used the Qiagen OneStep RT-PCR Kit, performed in a total volume of 25 µl and template volume of 5 µl. In the PCR were used primers A 11 and A14, and probe TaqMan Probe–FAM–Tamra. PCR studies were carried out with a thermo cycler machine "Applied Biosystems 7300 Real Time PCR System" with the temperature control for reverse transcription at t = +50 °C – 30 min, inactivation of reverse transcriptase and activation of Taq at t = +95 °C – 15 min, denaturation at t = +95 °C – 10 min, extension at t = +60 °C – 30 min for 40 cycles.

Studying the antimicrobial action of the catholyte/anolyte

The catholyte and the anolyte were prepared with using the Activator-I device, developed at the Institute of Information and Communication Technologies at BAS (Bulgaria). For this, drinking water without aditional quantity of NaCl was used. This led to pH = 3,0 and ORP = +480 mV for the anolyte, and pH = 9,8 and ORP = -180 mV for the catholyte. The Colony Forming Units (CFU) technique was used in this study to assess cellular viability. The conditions for the bacterial cultures growth were as described in our previous paper [26]. The bacterial cells were cultivated on the LB-medium (pH = 7,5) with 1 % bactotryptone; 0,5% yeast extract; 1,0% NaCl at t = +37 °C. After overnight cultivation of bacteria 100 µl samples of culture liquids were taken, centrifuged for 1 min at 10000 g and the pellet of bacterial cells was resuspended in 100 µl of the anolyte or the catholyte. As control samples were used the bacterial samples, re-suspended in non-electroactivated water. Different dilutions of cells were spread on LB-agar Petri plates. After the overnight incubation at t = +7 °C the appeared bacterial colonies were counted. The viable cells

were calculated as a percentage from the CFU. The CFU obtained from culture liguids treated with non-electrochemically activated water were accepted as 100 %.

Spectral analysis of the catholyte/anolyte by NES and DNES methods

The NES and DNES methods were used for the estimation of energy of hydrogen bonds of the anolyte, the catholyte and deionized water in order to make a supposition about the spectrum characteristics and structural changes. The device measures the angle of evaporation of water drops from 72^0 to 0^0. As the main estimation criterion was used the average energy ($\Delta E_{H...O}$) of hydrogen O...H-bonds between individual H_2O molecules in water's samples. The NES-spectrum of water was measured in the range of energy of hydrogen bonds 0,08–0,387 eV or λ = 8,9–13,8 μm with using a specially designed computer program.

Results and Discussion

Research into the effects of electro-activated aqueous NaCl (the anolyte)on the CSF virus

As shown in Figure 2 the cytoplasm of cells infected by the CSF virus was stained in the dark reddish brown color (positive reaction), whereas in the uninfected cells it was colorless. That indicates on the presence of viral antigen in the samples.

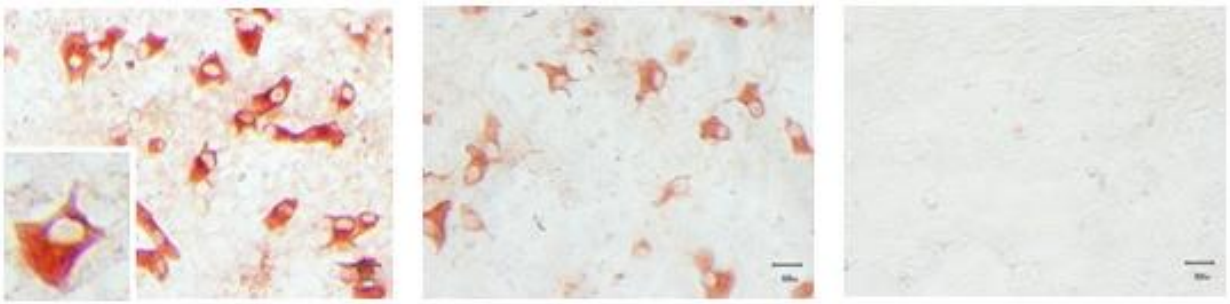

Figure 2: The established presence of viral antigen in cell cultures (left) and a negative control (right).

Table 1 summarizes the results of different experiments of the virucidal action of the anolyte on the cell culture suspension of the CSF virus upon infecting cell monolayer PK-15. As is shown in Table 1, upon the treatment of the viral inoculum with the anolyte in a 1:1 dilution, there was no viral growth in the four infected wells of the plate; upon 1:2 dilution there was no growth in two of the wells, the other two were reported as positive. Upon the treatment with the anolyte in dilutions 1:3 and 1:4, the result was identical: no growth in one of the contaminated wells of the plate, and poor growth – in the other three. The results obtained by the infection of the CSF virus a cell monolayer PK-15 and cell suspension were identical.

Table 2 summarizes the results of studies aimed at the evaluation of the virucidal effect of the anolyte on organ suspension containing CSF virus upon infecting a cell monolayer PK-15 with the virus. According to the data, upon treatment of the CSF viral inoculum (organ suspension) with the anolyte in all dilutions, there was no viral growth in the four infected wells of the plate.

Table 1: The virucidal action of the anolyte on cell culture suspensions of the CSF virus upon infecting a cell monolayer PK-15

Contamination of CC with:	Dilutions of anolyte (100 μl)	Total volume of the inoculum (μl)	Concentration of anolyte in %	Number of wells:	Result: positive/negative:
Virus 200 μl	–	200	–	4	4/0
Virus 100 μl	1:1	200	25	4	0/4
Virus 100 μl	1:2	200	16,51	4	2/2
Virus 100 μl	1:3	200	12.5	4	3/1
Virus 100 μl	1:4	200	10	4	3/1

Table 2: The virucidal action of the anolyte on organ suspensions containing CSF virus upon infecting a cell monolayer PK-15

Contamination of CC with:	Dilutions of anolyte (100 µl)	Total volume of the inoculum (µl)	Concentration of anolyte in %	Number of wells:	Result: positive/negative:
Virus 200 µl	–	200	–	4	4/0
Virus 100 µl	1:1	200	50	4	0/4
Virus 50 µl	3:1	200	75	4	0/4
Virus 25 µl	7:1	200	87	4	0/4
Virus 12,5 µl	15:1	200	94	4	0/4

Evidently, the anolyte has a destructive influence on the envelope of the CSF virus, wherein the main antigens (proteins) are localized. Studies of the viral inocula used in the tests by means of polymerase chain reaction (PCR) in real time demonstrated the presence of a genome (RNA) in them, also after the treatment with the anolyte. Some shortening of the time was proved (the decreased number of amplification cycles), required for the formation of a stable fluorescent signal, respectively, a positive reaction for a genome, is closely correlated with the exposure under the treatment of the viral inocula. The longer the exposure of processing with the anolyte, the sooner the presence of the viral RNA in the PCR was detected. According to one of our co-authors (Stoil Karadzhov), this may serve as an indirect indication that the anolyte destroys the CSF virus envelope, which, in its turn, facilitates the extraction of viral RNA and its more rapid reading by the fluorescent signal. However, there is still no sufficient convincing evidence on the impact of different concentrations of the anolyte on CSF viral particles. The analogous experiments carried out by Russian and German researchers were carried out mainly with the concentrated anolyte. The maximum virucidal effect detected in those experiments confirmed a strong virucidal action of the electrochemically activated aqueous solution of NaCl on the CSF virus. The difference in the results evidently is due to the use of lower concentrations of NaCl in our experiments. We attributed the essential significance to the fact that we determined the concentration limit (25 %) of the well demonstrated by the virucidal activity. In this aspect the further studies on reducing the time of the virucidal action, and the conducting of experiments in the presence of biofilms which protect viruses would be promising.

Research into the antibacterial effects of the anolyte and the catholyte on a strain of *E. coli DH5a*

In order to assess the effect, if any, of the electrochemically activated NaCl solutions (catholyte/anolyte) on bacterial cells we treated the cultures of a strain of *E. coli DH5a* by the catholyte and the anolyte. After the treatment of bacterial cells the colonies appearing on the plates with 2 % agar were obtained, produced by survived cells, which were further counted by the CFU method. Therefore, the number of colonies was presented on Figure 3 as a percentage of viable cells. It can be seen from Figure 3 that bacterial cells of the strain of *E. coli DH5a* treated with the catholyte were hardly survived after the treatment with only approximately 15% of the cells being survived. This result clearly shows that the electrochemically activated NaCl solution produced at the cathode possesses a strong bacteriocidal activity on the strain of *E. coli DH5a*. Thus, the catholyte with ORP ≈ -180 mV and pH = 9,8 demonstrated the better biocidal effect than the anolyte with ORP ≈ +500 and pH = 3,9.

Notably, the anolyte with ORP ≈ +500 and pH = 3,9 also showed slight antibacterial effect. Thus, approximately, 73% of the bacterial cells of the stain of *E. coli DH5a* were survived after the electrochemical treatment with the anolyte. In summary, it is assumed that both types of the electrochemically activated water solutions (catholite/anolyte) possess antibacterial effect on the strain of *E. coli DH5a*, however it is obvious that the catholyte has a stronger bacteriocide effect than the anolyte.

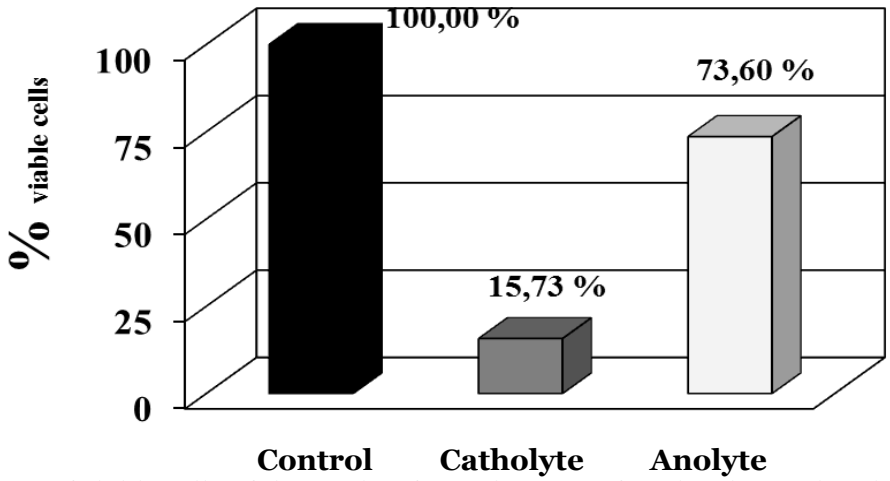

Figure 3. Percentage of viable cells of the strain of *E. coli DH5a* after the electrochemical treatment with the catholyte and anolyte relative to the non-electrochemically activated water

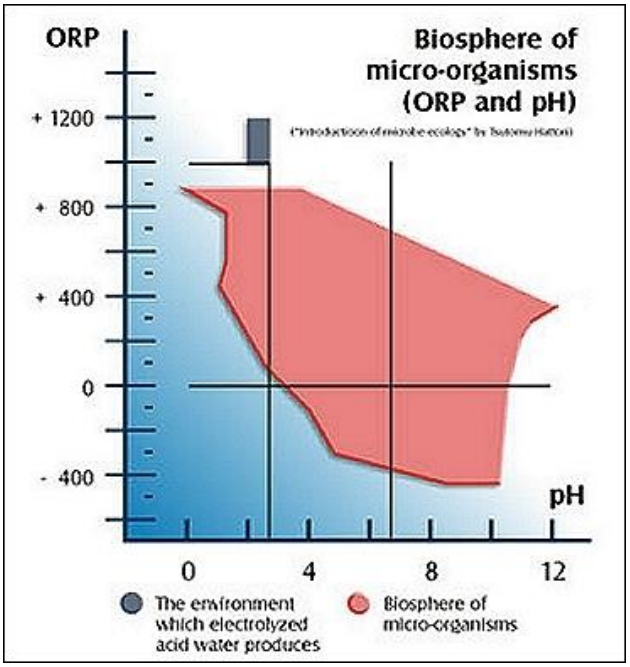

Figure 4. The dependence between the acidity and basicity (pH) of electrochemically activated solution of NaCl and the oxidation-reduction potential (ORP) on the biosphere of microorganisms

Figure 4 shows the dependence between the acidity and basicity (pH) of the electrochemically activated solution of NaCl and the oxidation-reduction potential (ORP). The pH value within the interval from 3 to 10 units and the ORP within the interval from -400 mV to +900 mV characterize the area of the biosphere of microorganisms. Outside these ranges of the pH and the ORP values the microorganisms will hardly survive in electrochemically activated environments. The disinfecting effect in this case is strengthened by the residual chlorine in electrochemically activated solution of NaCl, destructing unsaturated fatty acids, phospholipids and protein in the cell membrane.

NES and DNES spectral analysis of the anolyte and the catholyte

Other method for obtaining useful information about the structural changes in water solutions and the average energy of hydrogen bonds is the measuring of the energy spectrum of the water state. It was established experimentally that at evaporation of water droplet the contact angle

θ decreases discretely to zero, whereas the diameter of the droplet changes insignificantly [27]. By measuring this angle within a regular time intervals a functional dependence *f(θ)* can be determined, which is designated as "the spectrum of the water state" (SWS) [28]. For practical purposes by registering the SWS it is possible to obtain information about the averaged energy of hydrogen bonds in an aqueous sample. For this purpose the model of W. Luck was used, which consider water as an associated liquid, consisted of O–H…O–H groups [29]. The major part of these groups is designated by the energy of hydrogen bonds (-*E*), while the others are free (*E* = 0). The energy distribution function *f(E)* is measured in electron-volts (eV^{-1}) and may be varied under the influence of various external factors on water as temperature and pressure.

For calculation of the function f(*E*) experimental dependence between the water surface tension measured by the wetting angle (θ) and the energy of hydrogen bonds (*E*) is established:

$$f(E) = \frac{14{,}33\, f(\theta)}{[1-(1+bE)^2]^2}, \qquad (8)$$

where $b = 14{,}33\ eV^{-1}$; $\theta = \arccos(-1 - bE)$

The energy of hydrogen bonds (*E*) measured in electron-volts (eV) is designated by the spectrum of energy distribution. This spectrum is characterized by non-equilibrium process of water droplets evaporation, thus the term "non-equilibrium energy spectrum of water" (NES) is applied.

The difference *Δf(E) = f (samples of water) – f (control sample of water)* – is designated as the "differential non-equilibrium energy spectrum of water" (DNES) [30].

The DNES-spectrum measured in milielectron volts (0,001 eV) is a measure of changes in the structure of water as a result of external factors. Figure 5 shows the characteristic NES-spectrum of deionized water made from 25 independence measurements performed in a period of one year.

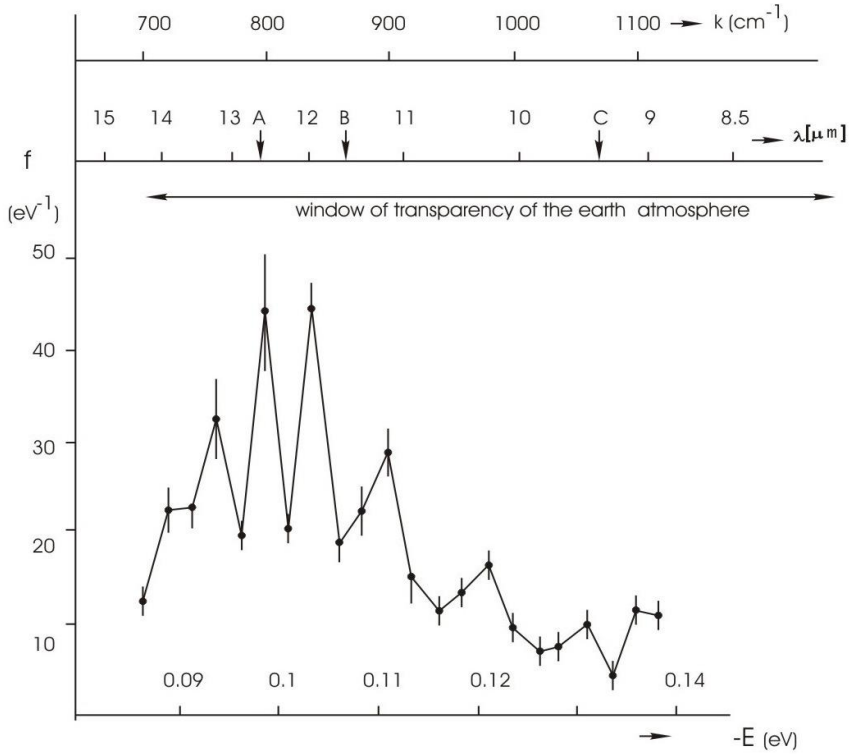

Figure 5. The NES-spectrum of deionized water (chemical purity – 99,99 %; pH – 6,5–7,5; total mineralization – 200 mg/l; electric conductivity – 10 μS/cm). The horizontal axis shows the energy of the H…O hydrogen bonds in the associates – E (eV). The vertical axis – energy distribution function – f (eV^{-1}). *k* – the vibration frequency of the H–O–H atoms (cm^{-1}); λ – wavelength (μm)

The average energy ($\Delta E_{H...O}$) of hydrogen H...O-bonds among individual molecules H_2O was calculated for the catholyte and the anolyte by NES- and DNES-methods. We studied the distribution of local maximums in catholyte and anolyte solutions. The local maximum for catholyte in the NES-spectrum was detected at -0,1285 eV, for anolyte – at -0,1227 eV, and for the control sample of deionized water – at -0,1245 eV. The calculations of $\Delta E_{H...O}$ for the catholyte with using the DNES method compiles (-0,004±0,0011 eV) and for anolyte (+1,8±0,0011 eV). These results suggest the restructuring of $\Delta E_{H...O}$ values among individual H_2O molecules with a statistically reliable increase of local maximums in DNES-spectra of the catholyte and anolyte (Table 3).

We tried to relate the antimicrobial and antiviral action of electrochemically activated NaCl with the characteristics of the DNES-spectra. For the catholyte the biggest local maximum was detected at -0,1387 eV, or at 8,95 μm. In 1992 A. Antonov performed experiments with the impact of different types of water on tumor mice cells. It was detected a decrease in the NES-spectrum compared with the control sample of cells from healthy mice. There was also a decrease of the local maximum at -0,1387 eV, or 8,95 μm in DNES-spectra. Notably, the local maximum at 8,95 μm was detected with the negative value. It should be noted that for the catholyte the local maximum in the DNES-spectrum was detected with the positive value at +133,3 eV^{-1}.

For the catholyte the biggest local maximum in the DNES-spectrum was detected at -0,1312 eV, or 9,45 μm. It should be noted that for the treatment of influenza in medical drugs is included $Al(OH)_3$ [31]. The local maximum in this case was measured at -0,1326 eV, or at 9,35 μm.

The evaluation of the possible number of hydrogen bonds as percent of H_2O molecules with different values of distribution of energies is presented in Table 4. These distributions are basically connected with the restructuring of H_2O molecules with the same energies. This serves as the base for evaluating the mathematical model explaining the behavior of the anolyte and catholyte regarding the distribution of H_2O molecules to the energies of hydrogen bonds [32].

Table 3: Local maximums of catholite and anolyte solutions in NES- and DNES-spectra

-E(eV) x-axis	Catholyte	Anolyte y-axis (eV^{-1})	Control sample y-axis (eV^{-1})	DNES Catholyte	DNES Anolyte	-E(eV) x-axis	Catholyte y-axis (eV^{-1})	Anolyte y-axis (eV^{-1})	Control sample y-axis (eV^{-1})	DNES Catholyte y-axis (eV^{-1})
0,0937	0	0	0	0	0	0,1187	0	66,7	66,7	-66,7
0,0962	0	0	0	0	0	0,1212	66,7	0	0	66,7
0,0987	0	0	0	0	0	0,1237	0	0	0	0
0,1012	66,7	66,7	33,3	33,4	33,4	0,1262	0	0	66,7	-66,7
0,1037	0	0	33,3	-33,3	-33,3	0,1287	0	0	66,7	-66,7
0,1062	0	0	0	0	0	0,1312	33,3	100	33,3	0
0,1087	0	0	0	0	0	0,1337	33,3	33,3	33,3	0
0,1112	0	0	0	0	0	0,1362	0	0	0	0
0,1137	0	66,7	66,7	-66,7	0	0,1387	200	66,7	66,7	133,3
0,1162	0	0	0	0	0	–	–	–	–	–

Table 4: Energy distribution in catholyte and anolyte solutions of electrochemically activated sodium chloride

-E(eV) x-axis	Catholyte y-axis, % ($-E_{value}$)/($-E_{total\ value}$)	Anolyte y-axis, % ($-E_{value}$)/($-E_{total\ value}$)	-E(eV) x-axis, % ($-E_{value}$)/($-E_{total\ value}$)	Catholyte y-axis, % ($-E_{value}$)/($-E_{total\ value}$)	Anolyte y-axis, % ($-E_{value}$)/($-E_{total\ value}$)
0,0937	0	0	0,1187	0	16,7
0,0962	0	0	0,1212	16,7	0
0,0987	0	0	0,1237	0	0
0,1012	16,7	16,7	0,1262	0	0
0,1037	0	0	0,1287	0	0
0,1062	0	0	0,1312	8,4	24,8
0,1087	0	0	0,1337	8,4	8,4

0,1112	0	0	0,1362	0	0
0,1137	0	16,7	0,1387	49,8	16,7
0,1162	0	0	–	–	–

Conclusion

The experimental results prove the strong influence of both types of electrochemically activated NaCl solutions (catholyte/anolyte) on microbes and viruses. They are in accordance with the results obtained by other researchers, and demonstrate the strong biocidal effect of the anolyte toward the CSF virus. Also, the interesting results on the antibacterial effect were obtained when a strain of *E. coli DH5a* was treated with the catholyte and anolyte, respectively. Unexpectidely, the catholyte with ORP ≈ -180 mV and pH = 9,8 demonstrated the better biocidal effect than the anolyte with ORP ≈ +500 and pH = 3,9. We tried to relate the antimicrobial and antiviral action of electrochemically activated NaCl solutions with the characteristics of the DNES-spectra. There is an indication about such a connection but more thorough research is needed to prove it. For example, the inverse biocidal effect between the catholyte and anolyte in case of the strain of *E. coli DH5a* requires a clear explanation.

The main results of the research are formulated as follows.

1. The anolyte did not affect the growth of the cell culture PK-15;
2. The anolyte administered at a concentration of 25 %, exerts a strong virucidal effect on a cell culture virus, and a weaker antiviral activity at concentrations of 16,51 %, 12,5 % and 10 %;
3. The anolyte exerted a strong virucidal effect at concentrations of 50 %, 75 %, 87 % and 94 % on the CSF virus in cell culture suspensions;
4. The catholyte supresses the growth of *E. coli* up to 85% while the anolyte is at least three times less effective;
5. The local maximum in the DNES-spectrum of the catholyte was detected at 9,85 μm; there was a decrease of this local maximum in water with mice tumor cells;
6. The local maximum in the DNES-spectrum of the anolyte was detected at 9,45 μm; at 9,35 μm it is occurred the effect of inflammation from virus of influenza;
7. The mathematical model of the catholyte and anolyte regarding the distribution of H_2O molecules to the energies of hydrogen bonds was evaluated.

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Исследование антимикробного и антивирусного эффектов электролитических водных растворов хлористого натрия NaCl (анолит/католит) на кишечную палочку *E. coli DH5* и вирус классической чумы свиней (КЧС)

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Аннотация. В работе приводятся результаты антивирусного и антимикробного действия электролитических водных растворов хлористого натрия (анолит/католит), полученных в анодной и катодной камере стандартной электролитической ячейки, на вирус классической чумы свиней (КЧС) и штамм кишечной палочки *E. coli DH5*. Показано, что анолит не влияет на рост культуры клеток РК-15; вирусной рост при заражении клеточного монослоя замедлялся вирусом КЧС в наибольшей степени при разведении анолита в пропорции 1:1 и менее в других разведениях; в то время как вирусный рост при инфекции клеточной суспензии с вирусом КЧС замедлялся анолита в наибольшей степени в разведении 1:1, и менее в других разведениях; вирусный рост при инфекции вирусом КЧС суспензии клеток монослоя зависел от присутствия анолита во всех разведениях. Неожиданно сильный бактерицидный эффект наблюдался при обработке штамма *E. coli*

DH5 анолитом и католитом соответственно. Для получения дополнительных данных об антибактериальной и антивирусной активности электроактивированных растворов NaCl, а также о структурных изменениях растворов католита и анолита, были измерены неравновесный энергетический спектр (НЭС) и дифференциальный неравновесный энергетический спектр (ДНЭС) анолита и католита.

Ключевые слова: анолит, католит, *E. coli DH5*, вирус классической чумы свиней, НЭС, ДНЭС.

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A Study of Psychosocial Risk Status and Knowledge of Reproductive Health in Adolescents in Raipur City

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Abstract

Adolescence is the period of physical and psychological development from the onset of puberty to maturity. The World Health Organization defines an adolescent as a person between ages 10 and 19 years. A dramatic shift in thinking from concrete to abstract gives adolescents a whole new set of mental tools, to analyze situations logically in terms of cause and effect, appreciate hypothetical situations, evaluate alternatives, introspection, decision making and cognitive abilities. Adolescents experience intense physical, psychological changes as they make transition from childhood to adulthood. This period of transition is the most vulnerable time. Adolescents are at risk of developing behavioral problems like school failure and drop outs, substance abuse, delinquency and violence, sexually transmitted diseases, unwanted pregnancies, domestic violence, stress and depression and risk-taking behaviour. It is the responsibility of the society to utilize this period constructively through education, counseling, mass media, awareness programmes and address the reproductive health needs of adolescents. The present study analyzes the psychosocial risk status and knowledge of reproductive health in adolescents in Raipur city. The study recommends implementation of provisions by government to provide continuous education and economic security for adolescents. Programmes like age-appropriate reproductive health curriculum should be introduced in schools and colleges with counseling facilities at school and primary health care levels.

Keywords: reproductive health, adolescents, contraception, sexuality, psychological development.

Introduction

Adolescence is the period of physical and psychological development from the onset of puberty to maturity. It is also referred to as teenage years or youth .Adolescence is defined by WHO as the age group of 10-19 years. In India, adolescents (10-19 years) constitute 21.4 percent of the population, comprising one-fifth of the total population. Adolescence is the transitional stage of development between childhood and adulthood, representing the period of time during which a

person experiences a variety of biological changes and encounters a number of emotional issues. Adolescence can be a specifically turbulent as well as a dynamic period of one's life. It has been identified as a period in which young people develop abstract thinking abilities, become more aware of their sexuality, develop a clearer sense of psychological identity and increase their independence from parents. G. Stanly Hall denoted this period as one of "Storm and Stress" and, according to him, conflict at this stage is normal and not unusual.

Adolescents report that they are far happier spending time with similarly-aged peers as compared to adults. Consequently, conflict between adolescents and their parents increase at this time as adolescents strive to create a separation and sense of independence. Young adolescents are particularly susceptible to conforming to the behavior of their peers. Adolescents are rich in memory, perceiving things, concept formation, association, generalization, imagination and decision making. Questioning on most of the things is prevalent. Adolescence is a period when rapid physiological changes and demands for new social roles take place. Emotional development is at its peak and there is no emotional stability in general. It is a period of rapid physical and psychological transition which makes adolescents particularly vulnerable to emotional conflicts and behavioral disorders.

Adolescents are facing multitude of problems throughout the world. Adolescents suffer from psychosocial problems at one time or the other during their development. The major psychosocial problems are substance abuse; internalizing disorders (depression, anxiety) and externalizing disorders (delinquency, aggression, educational difficulties).Many of these problems are transient in nature. Further adolescents may exhibit these problems in one setting and not in other (e.g. home, school).Several key transitional periods (moving from early elementary to middle school, moving from middle to high school or moving from high school to college) can present new challenges for these adolescents and symptoms of dysfunction may occur.

Nowadays, because of rapid industrialization and urbanization, majority of parents are employed and live in unitary set up, so unavoidably they get less time to look after their children. Under these circumstances, psychosocial and psychiatric problems are on the rise with increased risk-taking tendency and therefore susceptibility to behavioral problems at the time of puberty and new concern about reproductive health. Studies suggest that adolescents have limited knowledge about reproductive health, and know little about the natural processes of puberty, sexual or reproductive health. This lack of knowledge about reproductive health including the emerging threat of adolescence age unwanted pregnancies, sexually transmitted diseases and HIV/AIDS may have grave consequences for the country. Health problems of adolescents are very different from those of younger children and older adults. Due to lack of accurate information in the absence of proper guidance, adolescents are prone to various behavioral and reproductive health problems.

While the consequences of poverty do not spare the adolescent boys, the girls come through as the endangered sex. In the absence of general overall socioeconomic development, availability of safe drinking water, environmental sanitation and better access to public health care, including control of recurrent viral, bacterial and parasitic infections, nutritional and dietary supplementation are needed. There is a spate of work in the area of sexuality and reproductive health. Increase in the sexual activity, incidence of STDs/HIV and clinical abortions among unmarried adolescents are reported. Matters are further compounded by gender bias, myths and misconceptions associated with sexuality and reproductive health among adolescents combined with reluctance on part of the parents and schools to talk more freely about reproductive health. It is evident that fertility control through stringent implementation of legal age for marriage, nutrition and sex education, and protection of unmarried adolescent girls, demand urgent attention. Even though there is a large body of information on reproductive health, there is uneven coverage of studies on adolescents out of school as well as on the street related to their sexual practices. Studies on mental health, well being, and behavior problems among adolescents remain limited. The data suggest need for preventive, curative, and promotive measures dealing with mental health problems among the adolescents. Adolescents are the future of a nation. Their psychological well being is a responsibility of all including parents, teachers, health workers and policy makers with the view of fast modernization and increasing risk behavior. Unmet needs during this critical period have serious consequences not for the individual alone but for the family, community, society and nation at large.

Aims and objectives

1. To determine socio demographic profile of the study adolescent group population in Raipur city during July 2013 to June 2014.
2. To assess psychosocial risk status of adolescents (10 to 19 years) in Raipur city by WHO's 'HEEADSSS' method of evaluation during the study period.
3. To assess the knowledge of adolescents on reproductive health.
4. To make suitable recommendation on the basis of study.

Methodology

The present study has been conducted in the selected urban and slum areas of Raipur city, Chhattisgarh, India. According to 2011 consensus, the population of the Municipal Corporation area was 1010087. Effective literacy was 86.90%; male literacy was 92.39% and female literacy was 81.10%, sex ratio of 946 females per 1000 males. Climate tropical wet and dry climate. Moderate temperature remains throughout the year except March to June which can be extremely hot. The list of zones and its area was procured from the Municipal Corporation of Raipur; C.G. Sampling was done by multistage simple random sampling in which four zones were selected out of eight zones from the list of zones by lottery method. From each selected zone, four wards were selected randomly for study by lottery method. From each ward, one slum and one urban area were selected randomly. Thus, total 32 study areas were selected, 16 from slum and 16 from urban areas. Prevalence of adolescents was found to be 22.1% according to WHO 2011 data from which sample size was calculated by the formula:

$n=Z^2pq/L^2$

Where, z = 1.96, n = sample size = adolescents

Prevalence 22.1%, q = 100-p, L = permissible level of error in the estimated prevalence, taken as 15 (15% of 22.1 = 3.2)

The required sample size was calculated to be:

$n= 1.96*1.96*21.4*78.6/ (3.2)^2$ = 631 Adolescents.

Results and discussions

Study included 643 adolescents in which 321 boys and 322 girls divided into early, mid and late adolescence age 32.81%, 29.70% and 37.48% respectively. Adolescents were almost equally distributed in study areas with adolescents in slum 49.77% and urban areas 50.23% respectively. Majority of adolescents were in middle school 32.66%, followed by high and higher secondary 25.35%, 19.44%; rest were in primary school 14.46%, graduates 7.74% and illiterates 0.62%. Adolescents were 57.08% of class IV, 15.40% of class I, 14% of class II, 13.06% of class III and 0.47% of class V socioeconomic status. Out of 643 adolescents, 34 (5.29%) were employed in different kinds of works. Majority of study subjects were Hindus 92.38%, rest were Muslims 5.91%, Christians 1.09%, Sikhs 0.47%. Majority of them were of 1st and 2nd birth order 35.30%, 35.61% followed by 3rd birth order 18.51% and decreasing further. Adolescents' fathers education was equally distributed within the group with illiterate 15.40% and decreased 4.82%. Majority of them were unskilled 35.15% followed by professional and skilled, both nearly 18.5%. Mothers were mostly illiterate 31.10%, followed by primary and middle school education nearly 13.5%, and as level increases number of mothers getting higher education decreases. Mothers were mostly unemployed 74.34%, unskilled worker 13.69% and only 5.57% were professionals.

Risk Status

In this study, prevalence of psychosocial risk status in adolescents was found to be 30.48% at mild risk, 36.08% at moderate risk and 33.13% at severe risk among which males were more 18.04% at severe risk than females. Females were more at moderate risk 19.44% and 15.55% at mild risk. Only 0.31% adolescents were found to be normal. Late adolescents were maximum at risk with 37.48% in which majority was in severe risk 24.26%, than early 32.81% followed by mid adolescents 29.70%. Adolescents in majority were at moderate risk 36.08% in which mid adolescents were more in number 16.64%. Early adolescents were more at mild risk 21.15%, mid adolescents were more at moderate risk 16.64%, late adolescents were more at severe risk 24.26%. In study done by Paul S (2006) in adolescents of South West Delhi, 48.4% were at psychosocial risk

in which 16% were at moderate risk and 32.4% were at high risk which is almost similar to our study findings. In a study done by Gupta MK (2011), 40.0%, 43.6% and 11.75% study subjects were categorized as mild, moderate and severe risk for psychosocial problems respectively. A study was conducted by Ahmad A (2007) on prevalence of psychosocial problems among 390 school going male adolescents. The study revealed that the prevalence of psychosocial problems was maximum 25.2% in mid adolescents and minimum 10.3% in early adolescents. A cross sectional study was conducted by Kishore S (2009) among 840 adolescents in Dehradun. The results showed overall prevalence of psychosocial problems to be 31.2%. Psychosocial problems were more in males (34.77%) as compared to females (27.6%). Arun and Chavan (2009) in a study on 2400 students in Chandigarh found that 45.8% had psychological problems. In a study done by Chabbra GS (2012) more than one third (39.6%) adolescents were having psychological problems, 62% of male adolescents were having psychological problems in middle age group of 14-16 years in comparison to early (31.7%) and late (18%) age groups.

Home

Home contributes to 12.67% of the total risk status in this study. Violence at home strongly correlated with risk status of adolescents as found in our study. 29.39% experienced domestic violence at home and risk status goes on increasing with most in severe risk 18.97% and it was statistically highly significant. Similar findings were seen in study conducted by Chhabra GS (2012) in which 29% adolescents had experienced domestic violence. Solvenia study (2008) done by Sprah L showed bit lower percentage 18.7%. Also Lepisto (2010) showed much higher 67% parental violence against them.

Adolescents of lower socioeconomic status were more at risk as seen class I, class II were more in mild risk 7.47% and 6.69% were class III (at moderate risk 4.51%). Class IV and V were most in severe risk 23.17% and 0.32%. So, with lowering of socioeconomic status, psychosocial risk status goes on increasing. Almost similar finding was seen in study done by Ahmad A (2007), prevalence was found to be higher in lower socioeconomic class IV 30.8% as compared to class I 13.8%, class II 12.45% and class III 18.7%. A study conducted by Chhabra GS (2011) showed more prevalence in lower socioeconomic class 65.7% as compared to upper 18.8% and middle 11.6% socioeconomic class.

Education

Education contributed 19.23% to the overall psychosocial risk status in this study. Adolescents who were not going to school were more in severe risk than mid adolescents i.e. adolescents of middle and high school. Study done by Ahmad A (2007) showed contribution of education in psychosocial risk problems to be 17.4% with greatest prevalence in mid adolescence (14-15 years) 25.2%, followed by late (16-19 years) 18.6% than early adolescence 10.3%. Prevalence goes on increasing with decreasing socioeconomic status i.e. class I had 6.9%, class II 12.3%, class III 18.6% and class IV 30.8% respectively. Study done by Chhabra GS (2012) showed contribution of education in psychosocial risk prevalence to be 36.9%, much higher than this study.

Addiction

Out of 643 adolescents, 14.77% adolescents were addicted, majority in late adolescence 7.62%, 3.73% in mid adolescence and 3.42% in early adolescence. Among them, females were 4.20% mostly in late adolescence 2.95% and males 10.58% which increased from early to late adolescence. Adolescents residing in slums were addicted more 9.02% as compared to urban adolescents 5.72%. Female adolescents of slums were more addicted 2.95% than those of urban areas 1.24%. Majority were addicted to tobacco 58.45%, 30.28% took alcohol and 7.04% were addicted to some kind of drugs. Similar kind of finding was seen in study done by Prajapati M (2011), in which prevalence of addiction was found to be 15.9% among which tobacco was in majority 90.32% followed by alcohol 9.68%. In study done by Chhabra GS (2011), prevalence of addiction was found to be 53.1%, much higher than our study. In study done by Ahmad A (2007), prevalence of addiction was found to be 13.3% similar to our study, highest in late adolescents 20.9% than mid adolescents 18.7% and least 4.8% in mid adolescents.

Sexuality and Relationship

Sexual status and related knowledge contributed to 20.20% of overall prevalence of psychosocial risk behaviour. Adolescents in relation were 32.04%, out of whom 10.26% were sexually active. Correlation was found to be highly significant. Similar finding was seen in study done by Prajapati M (2011) in Ahmedabad, in which 13.45% of adolescents (22.64% girls and 77.36% boys) were sexually active pre-martially. A study done by Jejeebhoy S (2000), revealed 20 to 30% of boys and 0% to 10% of girls are sexually active pre-martially. A study conducted by Chhabra GS (2012) in Amritsar, Punjab revealed much higher 79.4% of adolescents were sexually active. Only 9.64% adolescents had knowledge of safe sexual practices, out of which most of them were from slums 10.94%, rest 8.36% were from urban area. Study done by Prajapati M (2011) revealed that out of 13.4% sexually active adolescents, only 35.85% had knowledge of safe sexual practices and used barrier contraceptives.

Suicidal Ideation

Adolescents thought of attempting suicide were 28.35%, out of which 24.73% actually tried to hurt or kill themselves. Among them, females 37.89% were more than males 18.75% who had ideation of suicide or hurting themselves. Similar finding was seen in study done by Siddharth T (2006) in which 25.4% females and 19.1 % males had suicidal ideation. Further 24.2 % females and 17.8% males, high suicidal ideation was seen in late adolescence with actual attempt to do it was 8.6% and 7.3% respectively. In a study done by Khurana S (2004), prevalence of suicidal ideation was found to be 25%, 16.6% planned for it and 8.3% attempted it. In the study of Chhabra GS (2012, prevalence of suicidal ideation was 20.4%, out of which 14% attempted it.

Puberty Knowledge:

Adolescents having knowledge that physical and mental changes occur during puberty were 50.86% and were equally distributed in male and female of both urban and slum study areas. Adolescents who knew about the pubertal changes were 28.77%. Knowledge was seen more in males than in females. In a study done by Agarwal S (2007), higher prevalence 76% was seen regarding physical signs of adolescence, out of which 18% thought that only menarche was the sign of puberty.

Adolescents knowing correct age of menarche were 64.23% with female predominance in both slum and urban study areas with almost equal distribution. In study done by Bobhate S (2011), majority 54.4% replied correct age of menarche to be 13-14 years. Similar finding was seen in study done by Singh SP (2006), in which knowledge about age of menarche at 12-14 years was 79%, which is higher than our study.

Adolescents 60.81% said that proper genital hygiene should be maintained daily. In a study done by Bobhate S (2011), 69.3% had knowledge of maintaining proper reproductive hygiene.

Pregnancy and Contraceptive knowledge:

In our study, 93.33% adolescents had knowledge that Oral Contraceptive Pills can prevent pregnancy, 3.89% did not have this knowledge. Regarding knowledge of emergency contraception, 50% of adolescents agreed that these are safe to use. 19.44% adolescents had doubt about the safety of emergency contraceptive drugs. 30.83% adolescents did not know which is safer.

Adolescents were interrogated regarding knowledge of different types of contraceptives. Majority replied barrier contraceptives (85.83%), followed by Emergency contraceptive pills 64.17%, Female sterilization 58.33%, Oral contraceptive pills 55.56%, Intrauterine device 34.17%, Withdrawl method 26.94%, Spermicidal jellies and foams 12.78%.

In the study done by Patanwar P (2013), 64.5% had knowledge of contraceptive methods. 35.4% adolescents did not have knowledge regarding Barrier contraceptives (8.9%), Intrauterine devices (2.7%), Oral contraceptive pills (1.3%). In the study done by Muzammil K (2009), 65.47% adolescents had knowledge of contraceptive methods, out of which, majority had knowledge of Barrier contraceptives (61.07%), Oral contraceptive pills (49.64%).

STD Knowledge:

Adolescents were interrogated about the knowledge of signs and symptoms of sexually transmitted diseases (STDs) in male and female, 76% adolescents were not aware of pain, ulcer,

discharge; 14.72% adolescents were aware about the symptom of discharge, ulcer 11.11%, pain 5%. In the study done by Ahmad A (2007), only 9.2% adolescents had knowledge of STDs; rest of the 67.9% did not have knowledge and 22.8% did not respond. In the study done by Patanwar (2013), 84% adolescents had heard of STDs. Prevalence of knowledge about signs and symptoms, 8.5% replied pain, boils over genital region 7.5%, itching 3.9%.

On interrogating about the curability of HIV, 20.06% adolescents said yes, 16.08% said no and 63.14% said that they did not know. In a study done by Anjali S (2009), 33.4% adolescents believed that treatment can cure HIV, 45.9% said no and 20.7% did not know. On asking if HIV can be tested, 19.91% said yes, 9.33% said no, majority 70.76% did not know. In the study of Muzammil K (2009), 85.95% adolescents had knowledge of AIDS. On asking whether HIV can be prevented, 49.61% said yes, 6.60% said no, 44.63% did not know.39.81% adolescents said HIV can be prevented by using barrier contraceptives, avoiding commercial sex worker 19.28%, avoiding used needle 16.02%, avoiding injectable drugs 9.95%, abstinance 1.40%.

For seeking reproductive health related queries, majority of adolescents said through television 80.25%, magazines and friends 51.4%, internet 44.63%, doctor's guidance 10.26%. Only 13.53% adolescents said that they will seek from their parents, out of which 12.75% preferred mother. In the study done by Ahmad A (2007), major source of information for adolescents were television 40.2%, which is less than our study, friends and peers 16.5%, radio 5.1%, parents 0.5%. In the study of Agrawal S (2007), 37.6% adolescents said electronic media and friends 17.6%. In case of reproductive health related problems, 38% said they will go to their parents, 32.8% to doctors, 26% to friends and only 1.6% to teachers.

Reproductive health teaching is necessary in school education according to 74.81%. In study done by Agrawal S (2007), 90% wanted reproductive health education should be provided at school. In study done by Patanwar (2013), 86.4% felt reproductive and sexual health should be added in school curriculum.

Table 1: Sex wise distribution of addicted adolescents according to their phase of adolescence

Addicted	Female				Male				Total			
	Yes	%	No	%	Yes	%	No	%	Yes	%	No	%
Early adolescence	4	0.62%	108	16.80%	18	2.80%	81	12.60 %	22	3.42%	189	29.39%
Mid adolescence	4	0.62%	91	14.15%	20	3.11%	76	11.82%	24	3.73%	167	25.97%
Late adolescence	19	2.95%	96	14.93%	30	4.67%	96	14..93 %	49	7.62%	192	29.86%
Total	27	4.20%	295	45.88%	68	10.58 %	253	39.35 %	95	14.77%	548	85.23%

Chi square = 9.819 d.f = 2 p = 0.007 Significant

Out of 643 adolescents, 14.77% adolescents were addicted majority in late adolescence 7.62%, 3.73% in mid adolescence and 3.42% in early adolescence. Among them, females were 4.20%, mostly in late adolescence 2.95% and males 10.58% which increases from early to late adolescence. The correlation was statically significant.

Table 2: Distribution of Psychosocial risk status according to adolescents Co education

Coeducation	Normal	%	Mild risk	%	Moderate risk	%	Severe risk	%	Total	%
Yes	2	0.31 %	176	27.37 %	178	27.68 %	114	17.73 %	470	73.09%

No	0	0.00 %	20	3.11%	54	8.40%	99	15.4%	173	26.91%
Total	2	0.31 %	196	30.48 %	232	36.08 %	213	33.12 %	643	100.00 %

Adolescents in co-education were more at mild 27.37% and moderate 27.68% risk, whereas adolescents not in co-education were seen more at severe risk i.e. 33.12%. The correlation was highly significant.

Table 3: Distribution of Psychosocial Risk Status according to Adolescents' Birth Order

Birth order	Normal	%	Mild risk	%	Moderate risk	%	Severe risk	%	Total	%
1	1	0.16 %	66	10.26%	80	12.44	80	12.44%	22	35.30%
2	1	0.16%	79	12.29%	79	12.29	69	10.73%	228	35.46%
3	0	0.00%	31	4.82%	52	8.09	36	5.6%	119	18.51%
4 to 7	0	0.00%	20	3.11%	21	3.27	28	4.36%	69	10.73%
Total	2	0.31 %	196	30.48 %	232	36.08	213	33.12%	643	100.00%

Chi Sq= 13.463 d.f =12 p = 0.336 Not significant

No significant correlation was seen among birth order of adolescents and prevalence of psychosocial risk status with maximum in moderate risk.

Table 4: Distribution of adolescents psychosocial risk status according to Mothers Education

Mothers' Education	Normal	%	Mild Risk	%	Moderate Risk	%	Severe Risk	%	Total	%
Illiterate	0	0.00 %	39	6.07 %	70	10.89	141	21.94	200	31.10
Primary	0	0.00 %	18	2.95 %	34	5.29	56	8.87%	89	13.84
Middle	0	0.00 %	24	3.73 %	35	5.44	37	5.75%	87	13.53
High	0	0.00 %	20	3.11%	28	4.35	40	6.22%	75	11.66
Higher Secondary	0	0.00 %	39	6.07 %	23	3.58	10	1.55%	70	10.89
Graduate	2	0.31 %	30	4.67 %	22	3.42	8	1.25%	61	9.49
Postgraduate	0	0.00 %	20	3.11%	15	2.33	9	1.41%	43	6.69

Died	0	0.00 %	6	0.93 %	5	0.78	9	1.22%	18	2.80
Total	2	0.31 %	196	30.64%	232	36.08	213	32.97	643	100.00

Chi Sq = 84.775 d.f=21 p = 0.0001 highly significant

Risk status goes on decreasing with increasing level of education of mothers with maximum prevalence of risk among illiterates 31.10. The correlation was statistically significant.

Table 5: Adolescents Risk Status in correlation to Violence at Home

Violence at Home	Normal	%	Mild Risk	%	Moderate Risk	%	Severe Risk	%	Tota1	%
Yes	0	0.00 %	18	2.80%	49	7.62%	122	18.97 %	189	29.39%
No	2	0.31 %	179	27.84 %	183	28.46 %	90	13.99 %	454	70.61%
Total	2	0.31 %	197	30.64 %	232	36.08 %	212	32.97 %	643	100.00 %

Chi Sq = 150.749 df = 3 p = 0.0001 highly significant

Adolescents those having violence at home were 29.39% among which most were in severe risk 18.97 %. Those having no violence at home were more at mild and moderate risk. The correlation was found to be highly significant.

Table 6: Adolescents Risk Status in correlation to their Working Status

Working	Normal	%	Mild Risk	%	Moderate Risk	%	Severe Risk	%	Total	%
Yes	0	0.00	2	0.31	2	0.31	30	4.67	34	5.29
No	2	0.31	194	30.17	230	35.77	183	28.46	609	94.71
Total	2	0.31	196	30.48	232	36.08	213	33.12	643	100

Chi Sq = 71.60 d.f = 3 p = 0.0001 highly significant

Adolescents who are working were found more in severe risk category 4.67 %, whereas those who were not working were more in mild and moderate risk. The correlation was found to be highly significant.

Table 7: Adolescents Risk Status in correlation to their Hobbies

Hobbies	Normal	%	Mild Risk	%	Moderate Risk	%	Severe Risk	%	Total	%
Yes	2	0.31	186	28.93	129	20.06	63	9.80	380	59.10
No	0	0.00	11	1.71	103	16.02	150	23.17	263	40.90
Total	2	0.31	197	30.64	232	36.08	213	32.97	643	100

Chi Sq= 183.33 d. f=3 p = 0.0001 highly significant

Adolescents having hobbies in majority were in mild 28.93% and moderate risk 20.06%. Adolescents not having hobbies were more in severe risk 23.17%. The correlation was found to be highly significant.

Conclusion

The present study is cross sectional, observational study conducted among 643 adolescents, out of which 321 male adolescents and 322 female adolescents were divided into early, mid and late adolescent age groups comprising 32.81%, 29.70% and 37.48% respectively. Adolescents were almost equally distributed in areas of slums 49.77% and urban 50.23% in Raipur city.

Majority of adolescents were at psychosocial risk, with 30.48% at mild risk, 36.08% at moderate risk and 33.13% were at severe risk, among which male adolescents were at greater risk 18.04% than male adolescents 15.1%.

Late adolescents were at greater risk with 37.48% than early adolescents 32.81%, followed by mid adolescents 29.70%. Adolescents in majority were at moderate risk 36.08%, out of which mid adolescents were more in number 16.64%. 33.13% of adolescents were at severe risk, out of which late adolescents formed majority 24.26%. Early adolescents were at mild risk 21.15%.

Adolescent risk status although almost equally distributed in urban and slum areas, but number of adolescents in slum areas were more in moderate and severe risk i.e. 19.75% and 19.59%, whereas urban adolescents were more at mild risk 20.06%.

Adolescents of middle school and high school were at greater risk 32.66% and 25.35% respectively. Adolescents of primary and middle school were at mild risk, adolescents of high school and higher secondary were at moderate risk and graduates were at severe risk.

Strong association was seen with adolescents of lower socioeconomic status. Class I and Class II were at mild risk 7.47% and 6.69%, whereas Class III were at moderate risk 4.51%. Class IV and V were mostly at severe risk 23.17% and 0.32% respectively. Thus, psychosocial risk status goes on increasing with lowering of psychosocial risk status.

Strong association was seen between psychosocial risk status and mother's level of education. Risk status goes on decreasing with increasing level of mother's education. Maximum prevalence of risk was among adolescents of illiterate mothers. Adolescents of co-education were at mild 27.37% and moderate 27.68% risk. But adolescents not in co-education schools were seen to be at severe risk i.e.15.4%. Adolescents those having violence at home were 29.39%, among which most adolescents were at severe risk 18.97%. Those having no violence at home were at mild and moderate risk.

Only 9.64% adolescents had complete knowledge of safe sexual practices. Adolescents with incomplete knowledge regarding safe sexual practices were in majority 90%, with 31.57% at moderate risk and 29.55% at mild risk. Only 55.83% adolescents had knowledge regarding prevention of pregnancy. Majority of adolescents knew about barrier contraceptives 85.83%, followed by Emergency contraceptive pills 64.17%, Female sterilization 58.33%, Oral contraceptive pills 55.56%. According to majority 29.44% adolescents, the best contraceptive method was barrier contraceptives, followed by Oral contraceptive pills 16.67% and Emergency contraceptive pills 14.17%.

Knowledge regarding symptoms and signs of Sexually Transmitted Diseases (STDs) was not satisfactory. Majority of them had knowledge of discharge from genital organs 14.72%, ulcer 11.11% and pain 5%.

Knowledge regarding HIV and AIDS was also unsatisfactory among adolescents. Only 19.91% adolescents had knowledge that HIV and AIDS can be prevented. Among these, majority of adolescents knew about barrier contraceptives 39.81% as a preventive method, followed by avoiding sex workers 19.28%, avoidance of used needles 16.02%, avoidance of inject able drugs 9.95%, avoidance of multiple sexual partners 1.40%. For seeking reproductive health related queries, adolescents rely in majority on 80.25%, on magazines and friends 51.4%, followed by internet 44.63%. Guidance of doctors was the choice of only 10.26% adolescents mainly by late adolescent age group. 13.53% would prefer to seek guidance from parents, out of which 12.75% preferred mother as compared to father. Radio was the least preferred method 0.31%.

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Upper Lid Coloboma – a Case Report

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Abstract

The upperlid colobomas are rare and usually associated with cryptophthalmos. The colobomas can either affect one or both eyes. They can be associated with life threatening syndromes which must always be looked out for. Our case however defied any of these associations.

Upper lid colobomas can lead to severe eyeball morbidities including blindness and must be attended to promptly either by conservative or surgical approaches depending on the size of the coloboma, the associated ocular morbidities and the age of the patient. The prognosis of the pathology is fairly good depending also on the surgeon's expertise and time of presentation of the patient to the surgeon.

Keywords: coloboma, repair, penthouse.

Introduction

Coloboma is a Greek word meaning removal of a part by mutilation [1, 2]. Colobomas could be congenital or acquired [3]. Typical eye colobomas are as a result of the failure of the choroidal fissure or fetal cleft to close during the developmental stage of the fetus. Colobomas can cause visual morbidity depending on the structures involved and how significant the defects are. Colobomas of the lids are rare and could affect either the upper or the lower lids or both lids, may be unilateral or bilateral and may be isolated or syndromic [3]. The management of the lid colobomas could be challenging depending on the extent of the defect, the visual affectation, associated eye diseases and the expertise of the surgeon. We therefore, present a 16-year-old female with a congenital, isolated upper eyelid coloboma that was managed with satiasfactory aesthetic result from our hospital.

Case report

A 16-year-old female presented in our clinic with a complaint of inability to close her right eye from birth. Pregnancy history was not available however; patient said she was told she was delivered at term with the defect. General examination revealed a generally stable patient

systemically. Maxillofacial examination revealed a full thickness defect of about one-third of the right upper eyelid (Fig. 1a and b)

Figure 1a. Coloboma of the upper eye lid

Figure 1b: Coloboma of the upper lid pre-op
(down gaze). (Straight gaze)

with mildly hyperemic conjunctiva in the right eye, clear cornea, normal anterior chamber, brisk pupil, normal iris, clear lens, pink disc with flat and complete retina in the right eye. No abnormality was detected in the left eye. However, the unaided visual acuities of the right and left eyes were 6/9 and 6/5 respectively. The IOPs were within normal limits (14mmHg and 12mmHg in the right and left eye respectively). The extra-ocular motilities were normal in both eyes; however, the left eye demonstrated lagophthalmos (Fig. 2).

Figure 2. Lagophthalmos with corneal sparing pre-op

Based on history and clinical examination an impression of congenital isolated upper eyelid coloboma was made.

Laboratory investigations including; full blood count, Urea and Electrolyte and Urinalysis were all within normal limits. The patient was subsequently slated for primary closure of the upper lid defect. After routine cleaning, draping and infiltration of the defect with 2% xylocaine local anaesthesia with 1 in 100,000 units of adrenaline, the defect was de-epithelized and fashioned a into a pent-house shape. Closure of the resultant defect was done in layer with 6/0 vicryl suturing for the inner lamella and 5/0 black silk suture for the outer lid lamella. The upper lid margin was anchored to the cheek skin as frost suture with uneventful recovery; silk sutures were removed seven days post-operatively (Fig. 3).

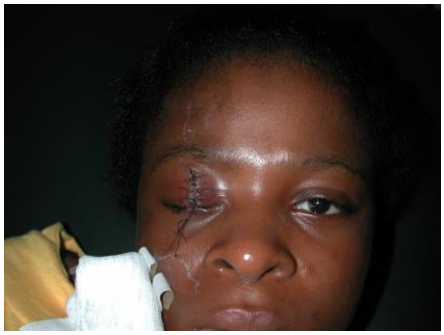

Figure3. Direct repair of coloboma- 24 hours post-op

The eye was padded for 24 hours and removed while the frost suture was left in situ for 72 hours only. Three weeks post –operatively patient was able to close and open the eyes with no lagophthlmos, however there was a drooping of the eye lid (Fig. 4) probably from the resolving lid edema inducing mechanical ptosis.

Figure 4. Near normal (mildly drooping) upper lid -3 weeks post-op

Discussion

The first description colobomas was by Jacques Guillemeu in 1585, thereafter John Mustarde observed that upper lid coloboma may be an isolated condition, associated with other facial anomalies or associated with some syndromes [4]. Colobomas of the eye ball and lids are either congenital or acquired. The typical isolated colobomas of the eyes are rare. The incidence of the typical eye colobomas is between 0.5-0.7 in 10,000 births [2]. The lid colobomas are commoner in the congenital colobomas. The mutation in the PAX2 gene seems more implicated in congenital eye colobomas. Colobomas of the eye could involve virtually all eye tissues including the lids, the iris, the choroid, the retina and the optic nerve. Acquired colobomas can be secondary to either surgical or non-surgical traumas.

Upper lid colobomas occur as a result of the failure of the lateral and nasal frontonasal mesenchymal processes of the optic cups of the optic vesicles to fuse together during the developmental stage of the embryo resulting in loss of the tarsal plate and muscularis layer of the upper lid. The lower lid colobomas are in turn as a result of the failure of the nasal and the lateral processes of the maxillary mesenchymal process to fuse together with the resultant loss of the lower lid tarsal plate and the muscular layer [5].

Typically eye ball colobomas hardly cause significant visual impairment except where there is significant colobomas involving a large portion of the retina and the optic nerve. The lid colobomas pose more challenges to the eyes. There is more tendency of losing the entire eye ball from exposure keratopathy.

The severity of eye morbidity due to upper lid colobomas is dependent on the grade of the coloboma. Nouby classified upper lid colobomas into 5 grades [6, 7] as follows:

- Grade 1- Coloboma without cryptophthalmos
- Grade 2- Coloboma with abortive cryptophthalmos
- Grade 3- Coloboma with complete cryptophthalmos
- Grade 4- Classical cryptophthalmos with absence of all eye lid structures and complete coverage of the eye by skin

• Grade 5- Severe cryptohthalmos with severe deformity of the nose and ectropion of the lip

Nouby suggested that upper lid colobomas, cryptophthalmos and facial deformities could be one anomaly [6]. Our case report did not have any associated facial deformities.

Lid colobomas ideally are not sight threatening except where the corneal is compromised as in very severe colobomas. Our patient only exhibited a mild visual drop of 6/12 unaided perhaps due to amblyopia, as compared with the normal (left) eye that had a visual acuity of 6/5 unaided. Visual loss in colobomas is difficult to resolve depending on the extent of damage.

Of all colobomas of the eye the lid colobomas are the commonest. The lid colobomas could either be acquired or congenital. A study conducted in the United States sometime revealed that of the 26 cases reviewed with congenital colobomas the upper lid colobomas were the commonest type of congenital colobomas [2]. Most upper lid colobomas are associated with cryptophthalmos and thus syndromic. The case presented here was isolated with no associated eye or systemic findings on gross examinations.

Syndromes associated with upper eye lid colobomas include; Fetal alcohol syndrome[3], a rare autosomal recessive condition characterized by eyelid colobomas, cryptophthalmos, anophthalmia or microphthalmia, an aberrant hairline, a bifid or broad nasal tip, gastro-intestinal anomalies or omphalocele and anal stenosis, the Charge syndrome[8] consisting of coloboma, heart disease, atresia choanae, retarded growth and development, genital hypoplasia, ear abnormality with deafness, the Fraser Syndrome, an autosomal recessive disorder with life expectancy of less than one year with features of cryptophthalmos, ear and nose defects, skeletal defect like syndactyly, urogenital and central nervous system defects, laryngeal stenosis, lung and liver defects, the renal coloboma syndrome [4] also known as the papillorenal syndrome, an autosomal dorminant syndrome due to mutation of the PAX2 gene characterized by optic nerve dysplasia and renal hypodysplasia with eye findings of microcornea, retinal coloboma, scleral staphyloma, optic nerve cyst and pigmentary macular dysplasia, wide excavated dysplastic optic disc with radial retinal vessels emerging from the disc margins directly, referred often to as the morning glory anomaly or the optic nerve coloboma and the Manitoba oculotrichoanal syndrome [9] which is a bilateral eyelid coloboma syndrome with bifid nasal tip hydrometrocolpos and vaginal atresia.

Treament of colobomas may be conservative or surgcal. The postion, severity of the coloboma, associated morbidities and syndomic involvement will determine the time and the type of intervention. Conservative or medical intervention include the use of lubricants, artificial tears, humid chambers and nocturnal padding [4, 7, 10] all in a bid ot avoid dry eye associated problems. The surgical modalities [4, 9, 10] depend on the size and thetype of the coloboma. Defects of about 1/3 or less can easily be closed by direct closure using the 2-layered approximation of the tarsus and the skin, after de-epithelising the edges of the defects[4, 9, 10]. This was the mode of treatment in the presented case here since the defect was about a third of the whole lid. Lateral cantholysis may be done in difficult cases [4, 10, 11].

For defects of 40% and 50% a 2-staged surgery is advocated [7, 12, 13]. The modified Cutler-Beard [7, 12, 13]. procedure in which the tarso-conjunctiva of the lower lid is fashioned to fill the upper lid defect while a free retroauricularis or the sliding skin grafts are used to cover the raw tarso-conjunctival surface for defects that are between 40% and 50%, while defects greater than 50%, the Tenzel or the modified Tenzel semi-circular lateral canthal flaps are used [7, 12, 13]. Other modalities that are applied in such huge defects include the Lateral Modified Hughes procedure and the full-thickness Lid rotation Flaps [7, 12, 13]

Conclusion

The upperlid colobomas are rare and usually associated with cryptophthalmos. The colobomas can either affect one or both eyes. They can be associated with life threatening syndromes which must always be looked out for. Our case however defied any of these associations.

Upper lid colobomas can lead to severe eyeball morbidities including blindness and must be attended to promptly either by conservative or surgical approaches depending on the size of the coloboma, the associated ocular morbidities and the age of the patient. The prognosis of the pathology is fairly good depending also on the surgeon's expertise and time of presentation of the patient to the surgeon.

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Comparison of Hemostatic and Neuro Protector Properties of Alkaloids N-Metiltsitizin and a Desoxypeganin in the Conditions of *in Vitro*

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Abstract

Modeling of sharp alcoholic intoxication induced animal 6 mg/kg of 50-70% ethanol by introduction. On this background studied effects of N-metilsitizin alkaloids and a desoxypeganin on ADP-induced aggregation of platelets and level of intracellular Ca^{2+} in the synaptosomes of a brain of rats. The obtained data render that the inhibiting effect of N-metilsitizin alkaloids and a desoxypeganin on ADP-induced aggregation of platelets is connected with oppression of a gain of cytoplasmatic concentration of Ca^{2+} from depot of platelets. Thus, N-metiltsitizin alkaloids and desoxypeganin block a gain of level of intracellular Ca^{2+} at the expense of increase in Ca^{2+} EPR pool, provoked by ethanol.

N-metiltsitizin doesn't compete with a glutamate for a binding site. Perhaps, action of N-metiltsitizin is caused by interaction with ionic channels of NMDA receptors. The neuronal of the receptors involved in the mechanisms which are the cornerstone of AAS (including convulsive attacks) and effectively to stop to possibility of application of N-metilsitizin in regulation of dihydropyridine-sensitive calcic channels of the main subtypes them. It is shown that the possible

competition between desoxypeganin and a glutamate for a site of binding of regulation of opening of ionic channels. Desoxypeganin directly doesn't affect calcic canals of a NMDA receptor. Perhaps, desoxypeganin like a glutamate causes overexcitation of NMDA receptors.

Keywords: desoxypeganin, N-metilsitizin, platelet, ADP, aggregation, argiolobate, synaptosome, ethanol.

Introduction

Ions of calcium are universal regulators and play the leading role in ensuring various cellular processes, including excitability of neurons, contractive activity of muscles, secretion of mediators and hormones, an expression of genes and proliferation of cages.

For realization of the majority of cellular processes in various cages certain Ca^{2+} a homeostasis in which regulation numerous Ca^{2+} the transporting systems of a cage take part is supported.

$[Ca^{2+}]$ in also intracellular depots – SR and EPR which have the specific Ca^{2+} -channels connected by IP3-and ryanodine receptors make an essential contribution to increase [1]. In all cases a moving force for providing transport of Ca^{2+} is the electrochemical gradient of Ca^{2+} which exists both between cytosols and extracellular space, and between cytosols and intracellular Ca^{2+} pools.

An important role in maintenance of intracellular Ca^{2+} of a -homeostasis plays also transporting Ca^{2+} - ATPase, localized on a plasmatic membrane and on membranes of intracellular organelle's. Thus low (100-150 nanometers) level of cytosolic Ca^{2+} at calm condition supported by active transport of ions of Ca^{2+} from cytoplasm in extracellular space with the help of Ca^{2+} ATPase of plasmatic membranes, and also mobilization of Ca^{2+} from cytosolic in intracellular compartment. Filling of intracellular pools happens at the expense of systems uni - and the anti-malt liquors localized in mitochondria and the secretory granules, and Ca^{2+} by ATPase of EPR and SR [2, 3].

It is quite obvious that the harmonious work of Ca^{2+}-transporting, which is provided both receipt of Ca^{2+} in a cage, and its removal from a cage, and also its mobilization in intracellular Ca^{2+} pools is the important factor providing maintenance of Ca^{2+}- of a homeostasis. In this regard violations of functional activity of separate links of Ca^{2+}- of the transporting systems often is followed by serious changes of level of intracellular Ca^{2+}, often is the main reason for pathogenesis of various diseases of cardiovascular and nervous systems. In particular, in warm and the smooth muscle leads increase in intracellular concentration of Ca^{2+} to change of electric properties of their membranes, violation of excitability and contractive activity. These changes, in turn, lead to development of such pathologies as a hypertension, stenocardia, coronary heart disease, a myocardial infarction and a stroke. At the same time, violations in work of Ca^{2+} of the transporting systems and a calcic overload of neurons are also the main reason for development of various pathologies of nervous system. In these conditions of change of electric parameters of membranes of neurons secretions of mediators and interaction between neurons are followed by violation of processes of generation and distribution of nervous impulses. Epilepsy, ischemia, various mental disorders, neuroses and depressions can be a consequence of such violations in neurons of a brain.

In this regard it is quite obvious that clarification of mechanisms of modulation of a calcic homeostasis and the related transport systems has not only important theoretical value, but also defines further development of the applied directions of biology and medicine.

One of the most actual problems of modern pharmacology and medicine is creation of new generation of medicines of the possessing highly effective and high-selective therapeutic action and not defiant serious side reactions. On the basis of the last achievements of combinatory chemistry, molecular biology, genetic engineering and biotechnology large-scale researches on working off of optimum strategy of the directed creation of new medicines are conducted. It is considered one of the main and defining principles of these strategy creations of bank of leading connections, structural and functional prototypes of future medicines. In this plan biologically active connections of a vegetable and animal origin the majority from which possess unique pharmacological properties and in centuries were used in traditional medicine, are considered as the most perspective for these purposes.

Similar versatility of pharmacological effects characterizes the majority of medicines of which under the action of interaction with ionic channels and neuroreceptors. This results from the fact that the same ionic channels, but to belonging functionally various cages and therefore the effects

caused by them aren't unambiguous can be a target for these connections. At the same time, it is revealed that some connections can work on various types of ionic channels and neuroreceptors [4]. So, Na^+ blocker channels disopiramide - can interact with a nicotinic cholinoretseptor. The blocker of K^+ of channels – amiodaron - is capable of inhibiting a muscarinic cholinoreceptor. Some blockers of Ca^{2+} channels can block also K^+ channels. Besides, it is shown that some blockers of NMDA of a receptor can interact with serotonins and adrenoreceptors. It is quite obvious that, possessing many-sided and ambiguous action on various types of cages and fabrics, the majority of these preparations along with positive therapeutic effect causes various undesirable side reactions. So, some antiarrhythmic means - Na^+ blockers channels cause increase of arterial pressure, violation of the speech, a depression and hallucinations. Some blockers of K^+ of channels applied as antiarrhythmic means [5], can also increase arterial pressure cause a depression of a hallucination and toxic affect a liver and lungs. α-adrenoceptors blockers can also cause violation of warm activity [6], bradycardia and a bronchospasm. Hypotensive preparations - blockers of Ca^{2+} of channels cause violation of warm activity, hearing and sight. Some blockers of NMDA of a receptor have negative effect on warm activity. Moreover, blockers of NMDA of a receptor can affect and receptors of adrenaline and serotonin and to cause various mental disorders and violations of physical activity [7].

It is known that abuse of alcohol causes violation of synthesis of various factors of the curtailing system, leads to decrease in its coagulative potential, developing of spontaneous bleedings, disseminates intra vascular folding's and malfunctions of platelets and a platelets link of a hemostasis [8, 9].

In preliminary researches it is established that at model rats both at sharp, and at chronic intoxication ethanol noted increase of ADP and adrenaline - the induced aggregation of platelets that testifies to increase of actually aggregation activity of platelets at alcoholic intoxication [10]. Perhaps, it is connected with activation of inductors of aggregation with increase in a gain of cytoplasmatic concentration of Ca^{2+} from depot of platelets.

At the same time chronic alcoholic intoxication causes compensatory increase in sensitivity of separate subunit of NMDA receptors, and also increases in density of receptors in various areas of a brain. At chronic effect of ethanol hyperproduction of NMDA receptors in membrane synaptosoma and the increase in their sensitivity connected with it to a glutamate is noted. The increase in an expression of NMDA receptors can be explained with the blocking effect of alcohol on the modulator glycine site and the subsequent compensatory increase in an expression of a receptor [11, 12].

The extremely dangerous consequences of alcoholism are established in activity of various parts of the nervous system where various mental disorders, depressions, epilepsy, and encephalopathy leading to degenerate disorders of cerebration, disability and disability develop. One of the neuronal of the mechanisms mediating sharp effects of ethanol is decrease in glutamatergic neurotransmission. Alcohol works as the antagonist of N-methyl-D-aspartate (NMDA) of receptors, one of subtype's glutamates the ionotropic receptors [12-14] that, undoubtedly, plays a role in the mechanisms which are the cornerstone of development of alcoholic intoxication and the alcohol abstinence syndrome (AAS) [15]. Clinical manifestations of AAS include psychopathological symptomatology, such as alarm, the depression, a dysphoria, irritability, sleep disorders and vegetative symptoms connected with the termination of alcohol intake [16, 17]. At chronic alcoholic intoxication ethanol cancellation, is followed, in particular, by the spasms connected with strengthening of the glutamatergic transfer happening at the expense of increase in release of a glutamate [18]. Antagonists of NMDA receptors in experiments on animals showed ability to block convulsive attacks during AAC [19]. Therefore it is supposed that the preparations blocking NMDA receptors or reducing glutamatergic neurotransmission by decrease in release of a glutamate can effectively stop AAS.

The solution of these questions occupied numerous scientific centers of the largest pharmaceutical companies, clinics and universities where leading experts not only pharmacologists and chemists, but also microbiologists, biochemists, electrophysiologists and biophysics are involved. In these centers on the basis of the last achievements of combinatory chemistry, molecular biology, genetic engineering and biotechnology large-scale researches on working off of optimum strategy of the directed creation of new medicines are conducted. It is considered one of the main and defining directions of this strategy the principle of creation of bank of leading

connections, structural and functional prototypes of future medicines. In this plan biologically active connections of a vegetable and animal origin the majority from which possess unique pharmacological properties, and for centuries were used in traditional medicine, are considered as the most perspective for these purposes.

Results of these researches are extremely important for establishment of new approaches of pharmacological regulation of Ca^{2+} a homeostasis in norm and at various pathologies nervous and platelet system and vascular.

The researches of pharmacological properties of vegetable alkaloids of a desoxypeganin and N-metilsitizin which are carried out at Institute of the Chemistry of Plant Substances, Academy of Sciences of the Republic of Uzbekistan on models of sharp alcoholic intoxication and chronic alcoholic dependence showed that among them there are perspective connections possessing potential tire-tread and anti-toxic action. However at what level their action is realized and what their molecular mechanisms remain not studied.

Research objective: Research action biological active substance on platelet and vascular, plasma links of a hemostasis and level of intracellular Ca^{2+} in the synaptosomes of a brain of rats, at the pathological changes observed at different conditions of alcoholic intoxication.

Materials and methods

Animals and Ethics statement: This study was carried out in the Laboratory Electrophysiology of Institute of Bioorganic Chemistry of Academy Sciences of the Republic of Uzbekistan on physically fit, adult, albino rats sexes (male) obtained from the vivarium of the Laboratory of Pharmacology. Animals had been fed with standard food and water in the vivarium. In all experiments albino rats weighing 200–250 g were used (n = 18). During the experiments, while working with experimental animals, International principles of the Helsinki Declaration and the rules of human attitudes towards animals were completely followed. Model experiments were made on 20 not purebred white rats males the weight (200-250 g) containing on a standard diet of a vivarium. All experiments carried out according to requirements of "The world society of protection of animals" and "The European convention on protection of experifcmental animals" [20]. Counted on each group of 20 rats background average daily consumption of 15% of ethanol for 1 kg of weight. To control group of animals in similar experimental conditions entered the distilled water. From them selected animals - chronic alcoholics in the period of mental and physical dependence on alcohol in the conditions of a free choice between 15% ethanol and water which is expressed in increase in a pathological inclination to alcohol. At modulation of sharp alcoholic intoxication the group of rats of alcoholic dependence was given 50-70% of ethanol.

Solvents and chemicals: Platelets allocated with a centrifugation method at 1500 rpm, within 15 min., for sedimentation of erythrocytes. The plasma enriched with platelets was centrifuged repeatedly within 10 min. at 3 thousand rpm. A deposit of platelets of a suspended in 5 ml of the environment containing *150 mm of NaCl, 2,7 mm of KCl, 0,37 mm of NaH_2PO_4, 1 mm of $MgCl_2$, 1 mm of $CaCl_2$, 5 mm glucose, 10 mm of HEPES-NaOH, pH 6,55, 50 of piece/ml of heparin, 0,35% of serumal albumine and 0,15 mg/ml of an apyrase*. Aggregation of platelets was registered on Born's method [21]. As inductors of aggregation of platelets used ADP (2 microns), adrenaline (5 microns) and thrombin (0,5 pieces/ml) (Sigma).

Synaptosomes allocated from a brain of rats with method of two-stage centrifugation [22]. All procedure of allocation was carried out at 4 °C.

For measurement of quantity of cytosolic Ca^{2+} ([Ca2 +] i) counted on Grinkevich's equation [23] synaptosomes, the rats allocated from a brain with chronic alcoholic intoxication placed on the middle, similar, that which was used for allocation of cages, were added by 20 microns of chlortetracycline (CTC). 60 min. for achievement of the maximum interaction of CTC with membrane-bound Ca^{2+}, as on plasmatic and intracellular membranes incubated. Wavelength of excitement of CTC – 405 nanometers, registration – 530 nanometers. Results expressed as a percentage, taking for 100% a difference between the maximum value of intensity of fluorescence (fluorescence of dye, saturated Ca^{2+}) and its minimum value (fluorescence of the indicator in lack of Ca^{2+}) received after addition EGTA ethylene glycol-encore-aminoetil-tetra acetate.

Aggregation on platelets was carried out on the photoelectric colorimeter (kfk-2), and also measurements of quantity of cytosolic Ca^{2+} in the synaptosomes were taken on the USB 2000 fluorimeter (Ocean Optics inc., First in PhotonicsTM. USB 2000. 2010. USA).

Data analysis the statistical importance of distinctions between controlled and skilled values was defined for a number of data, using the pair t-test where controlled and skilled values are taken together, and the unpaired t-test if they are taken separately. Value P<0, 05 indicated statistically significant distinctions. The received results are statistically processed on Origin 6.1 (Origin Lab Corporation, USA).

Results and discussion

Effect of alkaloid N - the metiltsitizin allocated from plants (*Thermopsis alterniflora*) and the desoxypeganin (2,3-trimetilen-3,4-digidrokhinazolina hydrochloride) allocated from plants of *Peganum harmala L* is investigated. On vascular platelets, plasma links of a hemostasis and level of intracellular Ca^{2+}in the synaptosomes of a brain of rats, at the pathological changes observed at different conditions of alcoholic intoxication.

At research of feature vascular platelets and plasma links of a hemostasis at model rats alcoholism, in a condition of sharp alcoholic intoxication of in vivo the blood plasma condensation is revealed. Thus thrombin (TT) and prothrombin time (PTT) in plasma with poor platelets was slightly extended, and also time of folding and formation of a fibrin clot in comparison with control (fig. 1). The mechanism of violation of folding of blood is a consequence of changes of a way as formations of fibrin, and its disintegration — a fibrinolysis. The end result of these violations at chronic alcoholism, despite all their complexity, there is a hypo coagulation which, possibly, is connected with a dysfibrinogenemia (qualitative defect of fibrinogen) or education and accumulation in a blood-groove of products of degradation.

However, in the plasma rich with platelets at model rats with chronic alcoholism of TT and PTT, on the contrary, folding time in comparison with control (fig. 1) is accelerated. Shortening of TT and PTT, as a rule, testifies to a hyperfibrinogenemia. In this case shortening of TT and PTT as earlier it was assumed, happens due to activation of factors of folding in a membrane of platelets that conducts to formation of the additional thrombin activating other coagulation factors (V, VIII, XIII, etc.).

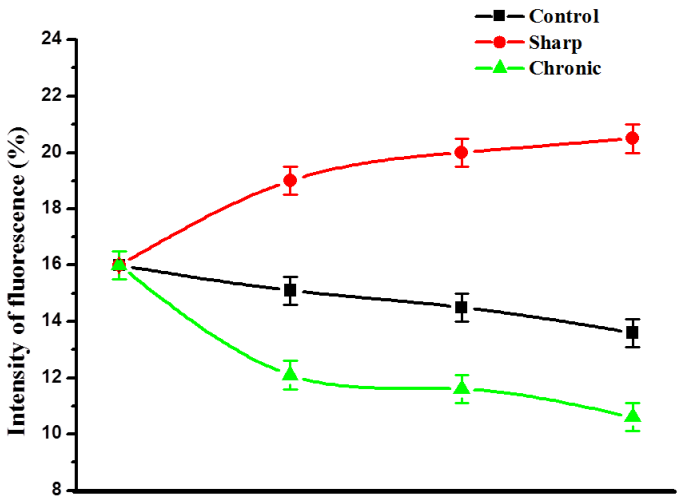

Figure 1. Influence of ethanol on factors of a fibrillation of model rats with chronic and sharp alcoholic intoxication. Reliability indicator: P<0, 05

At research of model rats with chronic alcoholism and in a condition of sharp alcoholic intoxication high rates of spontaneous aggregation of platelets are found. At research of plasma with rich platelets of blood of model rats with chronic alcoholism on adrenaline and ADP-induced aggregation also their dysfunction in response to the induced aggregation is revealed.

Dysfunction in response to the induced aggregation in plasma of blood of rats with chronic alcoholism, possibly, is connected with malfunction of receptors of membranes and secretion from platelets of activators of aggregation.

It is known that ADP and adrenaline are physiological inductors of aggregation and differ in mechanisms of activation of platelets. ADP, contacting glycoprotein receptors on a plasmatic membrane of a platelet, inhibits activity of an adenylate cyclase and by that reduces the cAMP level in cytoplasm, stimulates release of arachidonic acid and formation of a thromboxane of A_2 [24]. The effect of adrenaline on platelets is expressed much more weakly in comparison with ADP and activation of adrenoreceptors is connected with modulation of membranes and change of their permeability for ions of Ca^{2+}. Proceeding from above stated, it is possible to assume that effect of ethanol leads to decrease in level of cytosolic Ca^{2+} as a result of blocking of its entrance to a cage and its influence on activity of an adenylate cyclase and α-adrenoreceptor a plasmatic membrane.

When studying influence of ethanol on change of level of intracellular Ca^{2+} it was revealed that it causes dose dependent increase in intensity of fluorescence of CTC by 15-30% (fig. 2). It testifies that addition of ethanol leads to increase in quantity of Ca^{2+}, the rat associated with membranes of platelets.

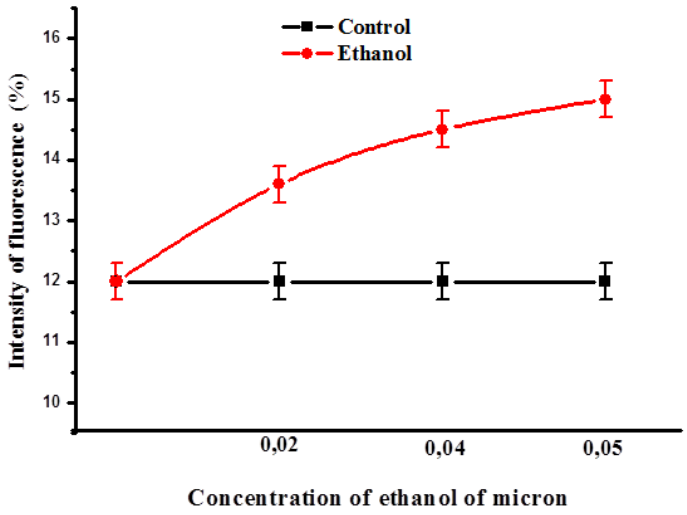

Figure 2. Influence of ethanol on change of level of membrane-bound Ca^{2+}.
As a probe CTC was used

It is known that the necessary level of ions of Ca^{2+} in cytoplasm is controlled by several systems of active transport of these ions through a plasmalemma and in intracellular Ca^{2+}depo (EPR and mitochondrion's). Thus, using the corresponding inhibitors, it is possible to estimate influence of ethanol on the size of intracellular Ca^{2+} pools. It is possible to judge the size of a reticular pool on effect of BHQ (a butylhydroxylchenon, inhibitor Ca^{2+}-ATPase EPR) which causes an exit of Ca^{2+}in cytosol that is registered as decrease in fluorescence of CTC.

In these conditions it was shown that effect of ethanol on the level Ca^{2+} in EPR had dose dependent character. Thus it is revealed that inhibitor Ca^{2+}- ATPase – BHQ caused bigger decrease in intensity of fluorescence of CTC in the cages processed by ethanol in comparison with control (fig. 3).

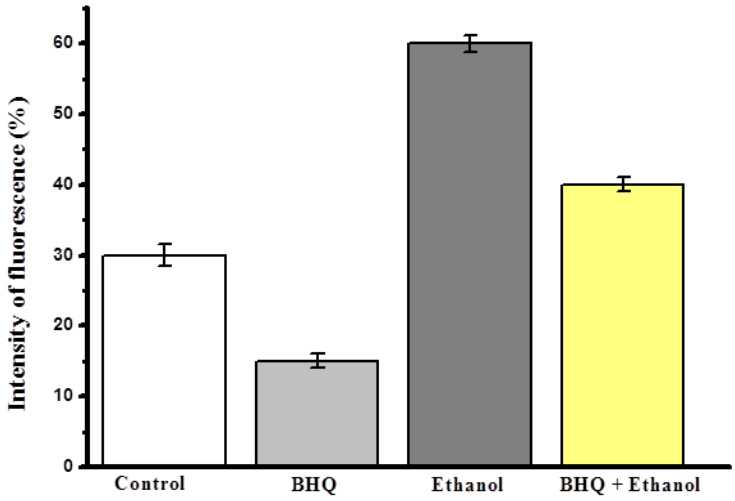

Figure 3. Influence of ethanol on intracellular Ca^{2+} a pool against BHQ. As a probe CTC was used. Reliability indicator: P<0, 05

So, in control decrease in intensity of fluorescence averaged 30%, and in the presence of 0,02-0,1 g/ml of ethanol – about 45-60%. It can be interpreted as increase in Ca^{2+} EPR pool, caused by inhibition of an exit of ions Ca^{2+} from a sarcoplasmic reticulum. Also it was shown that addition of inhibitors of breath (Rotenone) to the cages processed by ethanol doesn't cause considerable change of intensity of fluorescence of CTC. It allows assuming that ethanol has no essential impact on mitochondrial Ca^{2+} a pool.

Thus, at model rats with chronic alcoholism the activation of parameters of folding caused by the activation of a platelet hemostasis connected with violations of functional activity of platelets at the expense of increase in Ca^{2+} EPR pool that defines relevance of studying of mechanisms of a gain of cytoplasmatic concentration of Ca^{2+} comes to light.

At research of influence of N-metilsitizin alkaloids and a desoxypeganin against ethanol (0,05 g/ml) their dose dependent ant thrombin action is revealed. As ant thrombin action of a desoxypeganin and N-metilsitizin is shown more in the plasma rich with platelets, perhaps, their action is connected with secretion inhibition from platelets of activators of a fibrillation (a thromboxane of A2, ions of Ca^{2+}, the factor of activation of platelets (FAP), fibrinogen and many others) (fig. 4).

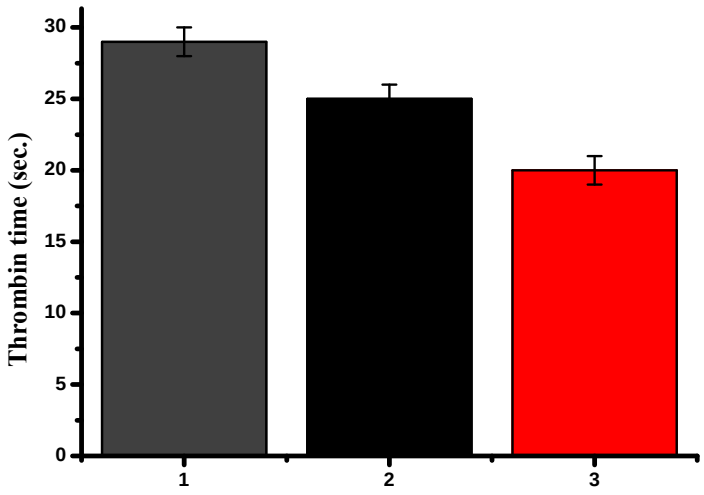

Figure 4. Dose dependent influence of alkaloids on a thrombin time
1 – Control; 2 - N-metiltsitizin; 3 – desoxypeganin. Reliability indicator: P<0, 05

At research of effect of alkaloids of a desoxypeganin and N-metilsitizin on functional activity of platelets it is revealed that N-metiltsitizin dose dependent inhibits adrenaline and ADP-induced aggregation of platelets. Thus the most inhibiting effect N-metiltsitizin alkaloid had, and its inhibiting properties are shown at ADP-induced aggregations. N-metiltsitizin at concentration of 50 microns causes 50% suppression ADP-induced of aggregation of platelets. Further increase of concentration of N-metilsitizin to 80 microns and 100 microns led to almost full inhibition ADP-induced of aggregation of platelets (fig. 5).

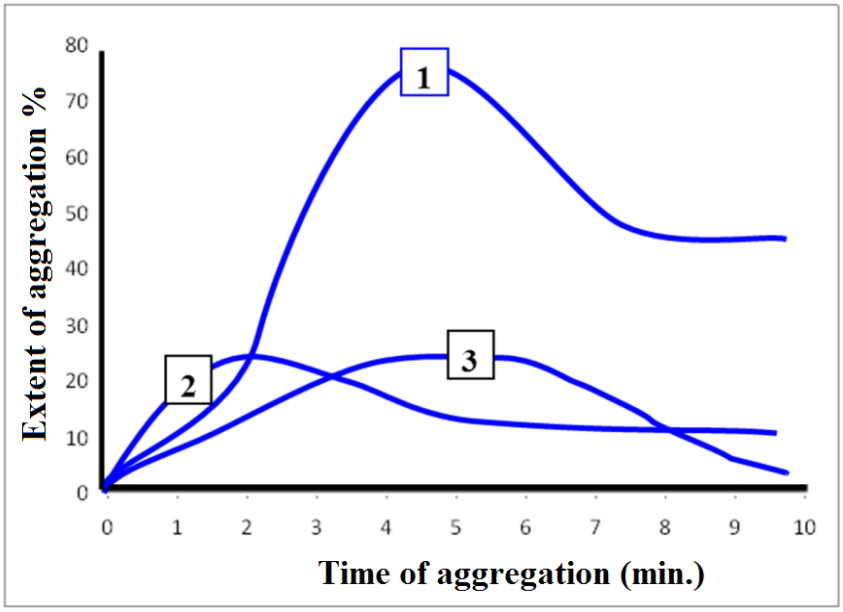

Figure 5. Influence of alkaloids on ADP-induced aggregation of platelets
1 – ADP-induced aggregation (control)
2 – ADP-induced aggregation against N-metilsitizin
3 – ADP-induced aggregation against a desoxypeganin

It is known that ADP leads to sharp increase in intracellular concentration $[Ca^{2+}]_i$, and this increase is carried out as at the expense of its entrance outside, and release from intracellular storages.

It is shown that against verapamil (a blocker of calcic channels) and a forskolin (the adenylate cyclase activator) in the concentration, for 50% reducing ADP-induced aggregation of platelets, inhibiting effects of N-metilsitizin and desoxypeganin amplified. The received results show that N-metiltsitizin alkaloids and desoxypeganin suppress activity of an adenylate cyclase and reduce level intracellular $[Ca^{2+}]_i$, perhaps their effects are connected with inhibition of a gain of cytoplasmatic Ca^{2+} as at the expense of its entrance outside, and release from intracellular storages.

With the purpose of specification of some mechanisms of antiaggregantis effect of N-metilsitizin alkaloids and a desoxypeganin their influence on the level of intracellular and membrane-bound Ca^{2+} with use of fluorescent probes Fura-2/AM and chlortetracyclin (CTC) was investigated. It is known that ADP leads to sharp increase in intracellular concentration $[Ca^{2+}]$. To define, whether effect of N-metilsitizin alkaloids and a desoxypeganin is based on a gain of cytoplasmatic concentration of Ca^{2+}, the induced ADP, experiment was made in the presence of physiological concentration of Ca^{2+} and without addition of Ca2 +. In control in both cases the fluorescence gain Fura-2/AM and CTC induced by ADP is revealed.

At research of effect of N-metilsitizin alkaloids and a desoxypeganin on a fluorescence gain Fura-2/AM induced by ADP in lack of extracellular Ca^{2+} it is revealed that N-metiltsitizin alkaloids and desoxypeganin dose dependent oppress release of Ca^{2+} from intracellular depots. Thus full suppression of a gain of cytoplasmatic concentration of Ca^{2+} wasn't observed. At the same time against N-metilsitizin alkaloids and a desoxypeganin, in the presence of extracellular Ca^{2+}, the fluorescence Fura-2/AM induced by ADP was much more, than in lack of extracellular Ca^{2+} that says that N-metiltsitizin alkaloids and desoxypeganin oppress only release of Ca^{2+} from intracellular depots. These assumptions are confirmed in researches of action of N-metilsitizin and

desoxypeganin against a blocker of Ca^{2+}- verapamil. Against N-metiltsitizin verapamil and desoxypeganin slightly oppressed a gain of level of intracellular Ca^{2+}, the induced ADP.

At linking of ADP with the corresponding receptors on a membrane of platelets, intermediate connections which stimulate release of calcium from depot are formed. At research of effect of N-metilsitizin alkaloids and a desoxypeganin against a forskolin (the adenylate cyclase activator) it is revealed that N-metiltsitizin alkaloids and desoxypeganin dose dependent strengthened the inhibiting action of a forskolin on ADP-induced increase of intracellular calcium.

In a case with use of fluorescent probes of CTC against N-metilsitizin alkaloids and a desoxypeganin considerable oppression of fluorescence of membrane-bound Ca^{2+} in lack of physiological concentration of Ca^{2+} was also observed. Perhaps, oppression of fluorescence of membrane-bound Ca^{2+} is connected with inhibition N-metiltsitizin alkaloids and desoxypeganin release of calcium from depot.

It is known that neurotoxic effects, and also some other manifestations of AAS are result of violation of balance between the inhibiting and exciting neurotransmitter systems and are partly mediated by glutamatergic neuromedia even system, change in particular of NMDA receptors and the level of intracellular Ca^{2+}.

The following work with synaptosomes of a brain of rats. Preincubation N-metiltsitizin alkaloids reduced fluorescence and respectively a level of cytosolic calcium at action of a glutamate on CTC-synaptosom's (fig. 6) complex.

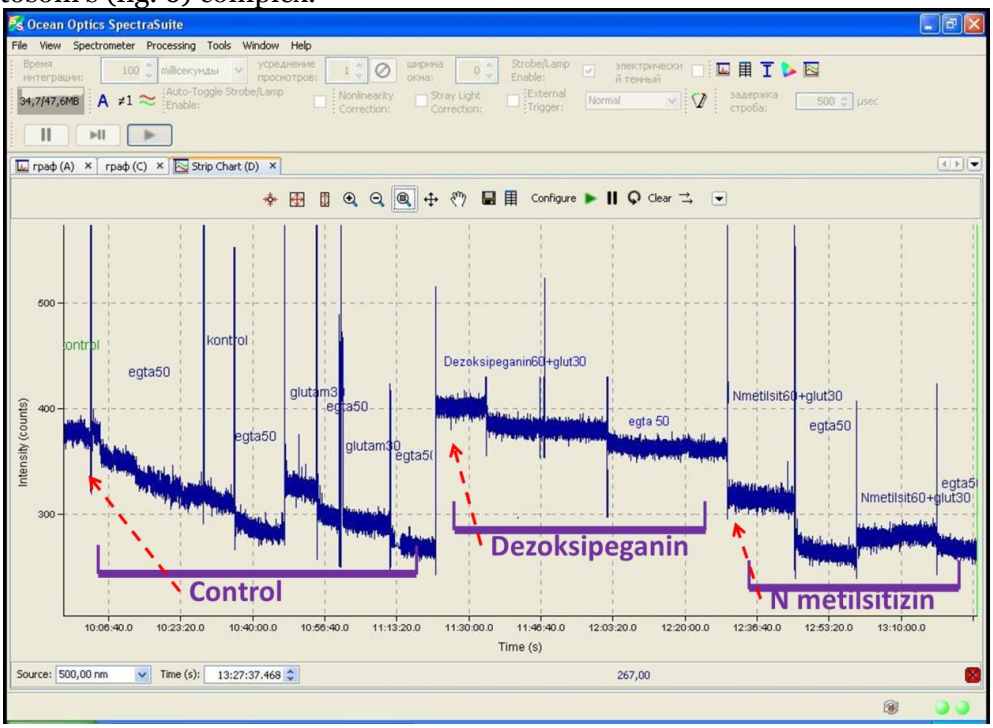

Figure 6. Influence of alkaloids of Desoxypeganin and N-metilsitizin on glutamatergic neuromedia even system at chronic alcoholic intoxication

At research of action of N-metilsitizin on synaptosoma of a brain of model rats with chronic alcoholic intoxication it is revealed that N-metiltsitizin considerably reduce fluorescence, respectively a level of cytosolic calcium in comparison with control. At the same time the increase in concentration of N-metilsitizin from 10 to 100 microns against a glutamate didn't lead to further decrease in effect of a glutamate.

The received results show that N-metiltsitizin alkaloids don't compete with a glutamate for a binding site. Perhaps, their actions are caused by interaction with ionic channels of NMDA receptors.

For identification, possible interaction of N-metilsitizin with sites of overexcitation of the NMDA receptors responsible for opening of calcic channels, their action against noncompetitive antagonists, such as magnesium ions, argiolobate and a blocker of the calcic channel – nifedipine is investigated.

In these researches it is shown that in the presence of N-metilsitizin the inhibiting action of ions of magnesium (10 microns) isn't observed. Possibly, it is caused by the competition between Mg^{2+} and N-metiltsitizin for sites stimulating opening of ionic channels.

Desoxypeganin at concentration of 10-50 microns differently influenced CTC-synaptosoma's complex. At a preincubation of a desoxypeganin with CTC-synaptosoma's complex of a brain of rats of control groups, its influence on fluorescence level isn't revealed. At the same time desoxypeganin induced decrease in fluorescence and respectively increase in level of cytosolic calcium in the presence of a glutamate in CTC-synaptosom's complex.

At research of action of a desoxypeganin on synaptosoma of a brain of model rats with chronic alcoholic intoxication it is revealed that desoxypeganin slightly increases fluorescence, respectively a level of cytosolic calcium in comparison with control (fig. 6). At the same time the preliminary preincubation of desoxypeganin (10 microns) synoptic membranes, and then led addition of the CTC-glutamate to decrease in fluorescence and respectively a level of cytosolic calcium. The Dose dependent increase in concentration of a desoxypeganin from 10 to 100 microns, led to dose dependent decrease in effect of a glutamate (fig. 7).

By results of the conducted researches it is possible to assume, the possible competition between of desoxypeganin and a glutamate for a site of binding of regulation of opening of ionic channels.

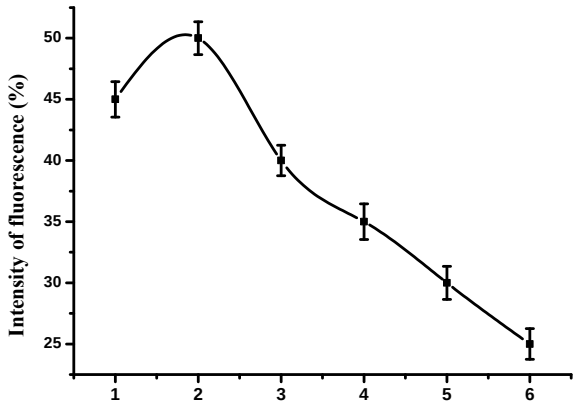

Figure 7. Influence of Alkaloid of a Desoxypeganin on Fluorescence and level of cytosolic calcium in the synaptosomes of a brain of rats

1 – Control (fluorescence of a complex of CTC-synaptosom) from a brain of rats at chronic alcoholic intoxication;

2 – Addition to CTC-synaptosom's complex of 10 microns of a desoxypeganin;

3–6 - action of a glutamate in concentration of 50 microns, against a preliminary incubation desoxypeganin in concentration 25, 50, 75, 100 microns

At research of action of N-metilsitizin on calcium - dependent processes of a NMDA receptor were studied against nifedipine (a blocker of Ca^{2+}-channels of L-type) in the synaptosomes from a brain of rats at a condition of AAS.

Preincubation of nifedipine with CTC-synaptosom's complex, led to decrease of fluorescence. Preincubation N-metilsitizin with CTC-synaptosom's complex, also led to decrease of fluorescence. Preincubation N-metilsitizin against nifedipine with CTC-synaptosom's complex, led to insignificant decrease of fluorescence (fig. 8) that points to the competition between alkaloids and nifedipine for a site of regulation of dihydropyridine-sensitive calcic channels.

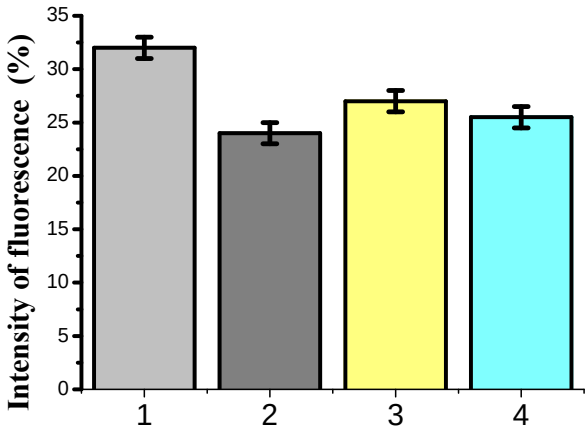

Figure 8. Influence of N-metilsitizin on calcium - dependent processes of a NMDA receptor against nifedipine

1 - Control (CTC-synaptosom's complex in conditions of AAS);
2 - nifedipin preincubation with CTC-synaptosom's complex;
3 - N-metilsitizin preincubation with CTC-synaptosom's complex;
4 - N-metilsitizin preincubation against nifedipine with CTC-synaptosom's complex;

It is known that glycine stimulates effects of a glutamate, and competitive antagonists of a receptor, such as AP_5, AV-2-1 toxin, can prevent activation of a glutamate. Other preparations and ions of Mg^{2+} can block the open channel by means of noncompetitive antagonism. To these preparations belong experimental neurotyre-tread preparation MK-801 and argiolobate [25].

To check whether it affects desoxypeganin to a site of linking of a glutamate with NMDA receptors or opening of the Ca^{2+}-channel oppresses, the following experiments are made.

Preincubation of alkaloid of a desoxypeganin with glycine shows stimulation of fluorescence and respectively strengthening of answers of a NMDA receptor that testifies that desoxypeganin like a glutamate causes overexcitation of NMDA receptors that leads to opening of calcic channels. Perhaps, desoxypeganin shows properties as agonist, and the antagonist.

For identification of possible interaction of a desoxypeganin with sites of overexcitation of the NMDA receptors responsible for opening of calcic channels, its action against noncompetitive antagonists – ions of magnesium and an argiolobatin is investigated.

It is shown that magnesium ions in the millimolar concentration considerably inhibit fluorescence of a complex glutamat-CTC-synaptosoma. The inhibiting action of ions of magnesium of fluorescence of a complex of CTC-synaptosom in the presence of a desoxypeganin didn't change. It is also shown that action of an argiolobatin on calcic canals of a NMDA receptor in the presence of a desoxypeganin doesn't change (fig. 9).

The received results testify that desoxypeganin directly doesn't affect calcic canals of a NMDA receptor. Perhaps, desoxypeganin like a glutamate, causes overexcitation of NMDA receptors.

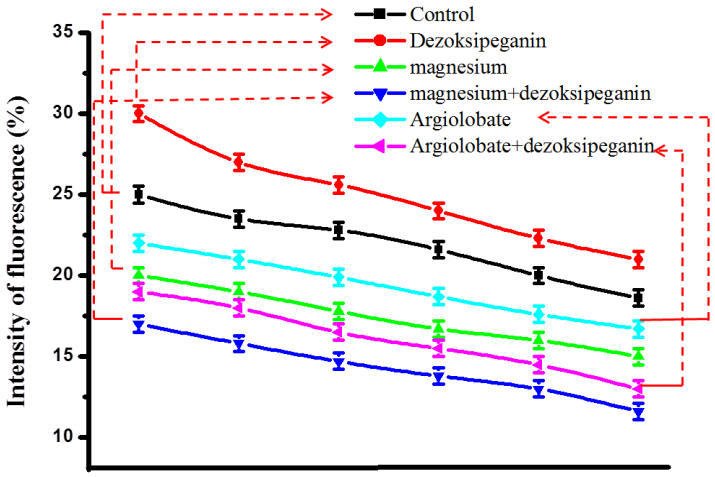

Figure 9. Influence of ions of magnesium, an argiolobatin and desoxypeganin on fluorescence and level of cytosolic calcium in the synaptosomas of a brain of rats

In the following experiments action of a desoxypeganin on synaptosoma of a brain of model rats with chronic alcoholic intoxication, after alcohol cancellation is investigated. In these researches it is shown that desoxypeganin considerably increases fluorescence, respectively a level of cytosolic Ca^{2+}, in synoptic membranes in comparison with control (fig. 10).

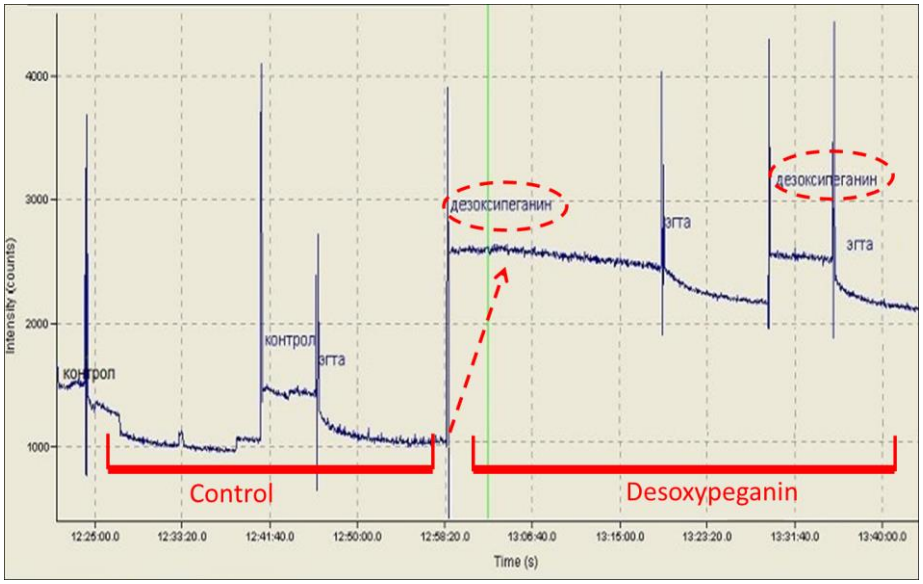

Figure 10. Influence of alkaloid of a desoxypeganin on fluorescence and level of cytosolic calcium in the synaptosomas of a brain of rats with chronic alcoholic intoxication, after alcohol cancellation

The received results testify that desoxypeganin directly doesn't affect calcic canals of a NMDA receptor. Perhaps, desoxypeganin like a glutamate causes overexcitation of NMDA receptors.

In case of ASS desoxypeganin considerably increases fluorescence, respectively a level of cytosolic Ca2 + and it can effectively be used as an exciting neurotransmitter preparation at chronic alcoholic intoxication.

Conclusion

The received results show that the inhibiting effect of N-metilsitizin alkaloids and a desoxypeganin on ADP-induced aggregation of platelets is connected with oppression of a gain of

cytoplasmatic concentration of Ca^{2+} from depot of platelets. Preliminary researches showed that ethanol at concentration of 0,01-0,03 mg/ml accelerates thrombin time of a fibrillation and causes spontaneous aggregation of platelets. Against N-metilsitizin alkaloid ethanol didn't influence process of a thrombogeneses and functional activity of platelets.

Thus, the received results confirm that alkaloids N-metiltsitizin and desoxypeganin block a gain of level of intracellular Ca^{2+}at the expense of increase in Ca^{2+} a pool of EPR provoked by ethanol.

The obtained results show that N-metiltsitizin alkaloids don't compete with a glutamate for a binding site. Perhaps, their actions are caused by interaction with ionic channels of NMDA receptors. The neuronal of the receptors involved in the mechanisms which are the cornerstone of AAS (including convulsive attacks) and effectively to stop to possibility of application of N-metilsitizin in regulation of dihydropyridine-sensitive calcic channels of the main subtypes them.

By the results of the conducted researches it is possible to assume, the possible competition between desoxypeganin and a glutamate for a site of binding of regulation of opening of ionic channels. The received results testify that desoxypeganin directly doesn't affect calcic canals of a NMDA receptor. Perhaps, desoxypeganin like a glutamate causes overexcitation of NMDA receptors.

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Сравнение гемостатических и нейропротекторных свойств алкалоидов N-метилцитизина и дезоксипеганина в условиях *in vitro*

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Аннотация. Моделирование острой алкогольной интоксикации индуцировали путем введения животным 6 мг/кг 50-70 % этанола. На данном фоне изучали действия алкалоидов N-метилцитизина и дезоксипеганина на АДФ-индуцированную агрегацию тромбоцитов и уровня внутриклеточного Ca^{2+} в синаптосомах мозга крыс. Полученные данные оказывают, что ингибирующий эффект алкалоидов N-метилцитизина и дезоксипеганина на АДФ-индуцированную агрегацию тромбоцитов связан с угнетением прироста

цитоплазматической концентрации Ca^{2+} из депо тромбоцитов. Таким образом, алкалоиды N-метилцитизин и дезоксипеганин блокируют прирост уровня внутриклеточного Ca^{2+} за счет увеличения Ca^{2+} пула ЭПР, спровоцированный этанолом.

N-метилцитизин не конкурируют с глутаматом за участок связывания. Возможно, действие N-метилцитизина обусловлены взаимодействием с ионными каналами NMDA-рецепторов. Возможности применения N-метилцитизина в регуляции дигидропиридин-чувствительных кальциевых каналов основных подтипов нейрональных рецепторов, вовлеченных в механизмы, лежащие в основе ААС (включая судорожные припадки) и эффективно купировать их. Показано что возможную конкуренцию между дезоксипеганином и глутаматом за участок связывания регуляции открывания ионных каналов. Дезоксипеганин непосредственно не действует на кальциевые каналы NMDA-рецептора. Возможно, дезоксипеганин подобно глутамату, вызывает перевозбуждение NMDA-рецепторов.

Ключевые слова: дезоксипеганин, N-метилцитизин, тромбоциты, АДФ, агрегация, аргиолобатин, синаптосома, этанол.

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The Inotropic Effects of 3'4'-Dimetyl Quercetin in Isolated Rat Papillary Muscle*

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Abstract

The aim of the present study was to determine the inotropic effect of 3'4'-dimethyl quercetin in the rat myocardium. Isometric tension forces were recorded using a force transducer (Type F30/Model D-79232; Hugo Sachs Elektronik, March-Hugstetten, Germany). 3',4'-dimethyl quercetin (10–100 μmol·L^{-1}) exerts a positive inotropic effect in rat papillary muscles. Thus, inhibitor potential-dependent $Ca^{2+}{}_L$-channels – nifedipine and inhibitor β-adrenoreceptor – propranolol has almost completely abolished the positive inotropic effects of 3',4'-dimethyl quercetin. Moreover, it was identified that the 3',4'-dimethyl quercetin depending on dose has increased quantity of the maximum velocity of force development, and the maximum velocity of papillary muscles relaxation. In conclusion, the present study demonstrates that 3',4'-dimethyl quercetin has showed a positive inotropic effect on rat papillary muscles that can be explained with the increase of $[cAMP]_{in}$ and may depend on increase of $[Ca^{2+}]_{in}$. Furthermore, lusitropic effect of 3',4'-dimethyl quercetin can be increased in cAMP and inhibition of phosphodiesterase enzyme activates protein kinase A, which phosphorylates regulator protein activity Ca^{2+}-ATPase – phospholamban and RyRs.

Keywords: papillary muscles, inotropic effect, 3',4'-dimethyl quercetin

Introduction

Nowadays, in pharmaceutical industry researches find cure against many various diseases, the direction of tendency is observed towards the growth of interest to search and create medical products based on bioflavonoids. Development of this field depends on having many advantageous

Abbreviations: AC – adenylate cyclase; ATP – adenosintriphosphat; $Ca^{2+}{}_L$-channels – the L-type Ca^{2+}–channels; $[cAMP]_{in}$ – the intracellular concentration of cyclic adenosine 3',5'–monophosphate; $[Ca^{2+}]_{in}$ – the intracellular concentration of Ca^{2+} ions; $+dF/dt_{max}$ – the maximum velocity of force development; $-dF/dt_{max}$ – the maximum velocity of relaxation; DMSO – dimethyl sulfoxide; EC_{50} – values of concentration for 50% of the maximal effect; Gs – guanine nucleotide-binding proteins; HSE – Hugo Sachs Elektronik; PKA – protein kinase A; SR – sarcoplasmic reticulum; RyRs – ryanodine receptors or sarcoplasmic reticulum Ca^{2+}–channels; CICR – "Ca^{2+} induced Ca^{2+} release"; SERCA – Ca^{2+}–ATPase of sarcoplasmic reticulum; $T_{contr.}$ – time from peak tension; $T_{1/2\ relax.}$ – time from to 50% relaxation.

pharmacological viewpoints of flavonoids. For example when bioflavonoids are utilized with other preparations, they will have pharmacological influence in wide spectrum with low toxicity, in addition to this it is identified that very seldom side effect occurred while practical absence of contra–indications.

Many flavonoids have low toxic influence on mammals and they show wide range of pharmacological influence and bioflavonoids are supposed to have a great therapeutic potential [1, 2]. According to previous experiments, flavonoids have a numerous valuable pharmacological impacts, such as cardiotonic, anticancer [3, 4], anti–atherosclerotic, antiproliferative, antiplatelet, antihypertensive [5], anticonvulsant, antibacterial and prophylactic effective preparations in various vascular diseases [2]. Many plant types containing flavonoids have been used in traditional Oriental medicine for thousands of years [6].

The flora of Central Asia is well known with very rich flora and various medical plants [7]. Moreover, scientists of Institute of the Chemistry of Plant Substances of Academy of Sciences of the Republic of Uzbekistan have extracted bioflavonoids from local plants [8, 9]. However, their mechanisms of pharmacological impact have not been studied yet. Therefore, the aim of the present study was to identify the possible mechanisms of the inotropic effect of 3',4'-dimethyl quercetin on the rat papillary muscle.

Material and methods

Animals and Ethics statement. This study was carried out in the Laboratory of Electrophysiology of Institute of Bioorganic Chemistry of Academy Sciences of the Republic of Uzbekistan on physically fit, adult, albino rats in both sexes (female and male) obtained from the vivarium in the Laboratory of Pharmacology. Animals had been fed with standard food and water in the vivarium. In all experiments albino rats weighing 200–250 g were used (n = 18). During the experiments, while working with experimental animals, International principles of the Helsinki Declaration and the rules of human attitudes towards animals were completely followed.

Solvents and chemicals. All reagents, which were used in experiments, were of analytic–grade (NaCl, KCl, $CaCl_2$, $MgSO_4$, KH_2PO_4, glucose, $NaHCO_3$), (±)-propranolol hydrochloride, prazosin, nifedipine hydrochloride, phentolamine, dimethyl sulfoxide (DMSO) were obtained from Sigma Chemical (St. Louis, Missouri, USA). 3',4'-dimethyl quercetin was produced from plants by scientists of the Institute of the Chemistry of Plant Substances of Academy Sciences of the Republic of Uzbekistan and presented by PhD Diloram ALIMOVA (Figure 1).

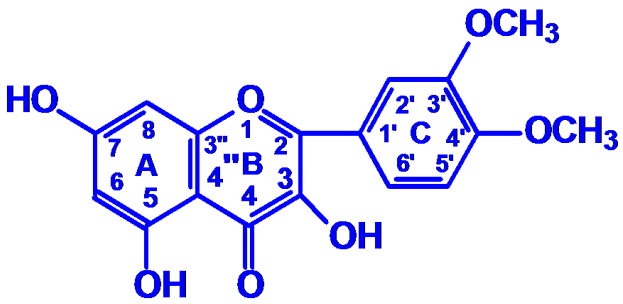

Figure 1. Chemical structure of 3',4'-dimethyl quercetin

3',4'-Dimethyl quercetin was categorized as a flavonol according to its structural complexity [6]. 3',4'-dimethyl quercetin and nifedipine hydrochloride were dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO did not surpass 0.1% under incubation condition and DMSO did not have any effect on the contractility of isolated papillary muscles preparations when added alone at a concentration of 0.1%. (±)-Propranolol hydrochloride was dissolved in deionized water in a concentration of 10 μmol·L^{-1}.

Preparation of tissue and measurement of contractility and setup of the equipment. The prepared papillary muscle was connected to a force transducer for signal recording. In experiments the papillary muscles preparations, isolated from the right atrium of adult albino rats' hearts. Rats were deeply anaesthetized with diethyl prior to paralyzing by using

cervical dislocation method. The papillary muscles were, 0.4–1.3 mm in diameter and 2.5–3.8 mm in length. The papillary muscles samples were prepared according to Sonnenblick (1964) [10], and the muscle was placed in an special horizontal tissue chamber (Type 813; Hugo Sachs Elektronik, March-Hugstetten, Germany), designed for the *in vitro* study in standard pharmacological experiments for measuring contraction force response of papillary muscle preparations. The top of the system was open and it is provided with the organ chamber, volume of 5 ml, the thermo–circulator for flow heater physiological solution and the wire holder for the force transducer (Type F30/Model D-79232; Hugo Sachs Elektronik, March-Hugstetten, Germany), with a precision micrometer control. In the experiments, modified the physiological Krebs–Henseleit solution containing (in mM): 118 NaCl; 4.7 KCl; 2.5 $CaCl_2$; 1.2 $MgSO_4$; 1.1 KH_2PO_4; 5.5 glucose and 25 $NaHCO_3$; pH 7.4 were used. This Krebs–Henseleit solution which was continuously bubbled with 95% O_2 and 5% CO_2 and kept at a temperature of +36±0.5 °C by means of water heating system controlled by temperature controller U8 (Bulgaria), and flowed in and out of the organ bath at a rate of 3–5 ml/min with the peristaltic pump LKB Bromma (Sweden).

The Isometric force transducer F30 is connected to a transducer amplifier (Type TAM-A; Hugo Sachs Elektronik, Harvard Apparatus GmbH, Germany). The papillary muscle was lifted with electric impuls that was higher than threthold (~20%), rectangular, electrical pulses of frequency 0.5 Hz; 5 ms and 5 V amplitude, delivered via a pair of platinum electrodes placed in the muscle–mounting organ chamber by using stimulator ESL–2 (Russia). Thus, wires of a pair of platinum electrodes were placed as parallel to the organ; the physiological solution of Krebs–Henseleit provides shortening the electrical contact distance between the electrodes and the preparate of the papillary muscles. In this experiment, 10 mN (1 g ~ 9.8 mN) was accepted for a resting tension of the preparation papillary muscles. After a 60 minute equilibration period, the length that provides development of the maximal isometric contractile force (L_{max}, the maximal length) length of the papillary muscle was found, and all experiments were carried out in these condition. After the equilibration period in the organ chamber, papillary muscles were stimulated by an initial electrical pulse of frequency 0.5 Hz, amplitude 5 V, and 5 msec pulses. The signals obtained were given from the transducer F30 to amplifier and sent to a computer by using a pen chart recorder (Type TZ 4620; Czech Republik) or a personal computer with analogue-digital converter LabPro Logger Lite 1.2 software (Vernier Software & Technology, Beaverton, USA).

In this experiments, the maximum velocity of force development ($+dF/dt_{max}$), and maximum velocity of relaxation ($-dF/dt_{max}$) of papillary muscles data were saved and analysed by means of specially software [11] running on a IBM PC computer interfaced with a D/A converter.

Data analysis. Papillary muscle contractions were plotted as a percentage of the force before the drug application in each muscle. Data were analyzed by OriginPro 7.0 (MicroCal Software, Northampton, MA). Pooled data are given as means ±S.E.M. of observations (n). Concentration–response curves were fitted to the logistic equation: $E = E_{\max} /(1+10^{-k\times([drug]-pD_2)})$, where E_{max} – is the maximal effect, k – is a factor which represents the slope of the curve, and pD_2 – is the drug concentration exhibiting 50% of the E_{max} expressed as negative log molar [12]. Values are expressed as mean ±S.E.M. Statistical differences of the data were calculated by ANOVA and the paired or unpaired Student's t–test where appropriate. The values were considered significantly different when $p<0.05$.

Results

The positive inotropic effects of 3',4'-dimethyl quercetin on rat myocardium. In the experiments, the 3',4'-dimethyl quercetin was added to the organ bath as the following concentration range: from 1 μmol·L⁻¹ to 100 μmol·L⁻¹ and doses were considered as the answer. Then 3',4'-dimethyl quercetin did not show inotropic effects at low concentrations (1–5 μmol·L⁻¹), and positive inotropic effects of 3',4'-dimethyl quercetin started to appear at concentrations 10 μmol·L⁻¹. And 3',4'-dimethyl quercetin (10–100 μmol·L⁻¹) showed dose-dependent positive inotropic effects (PIE) in rat papillary muscle contractility. Figure 2 shows the original recordings inotropic influence of 3',4'-dimethyl quercetin at 100 μmol·L⁻¹ concentration on the papillary muscle isometric contraction force (Figure 2).

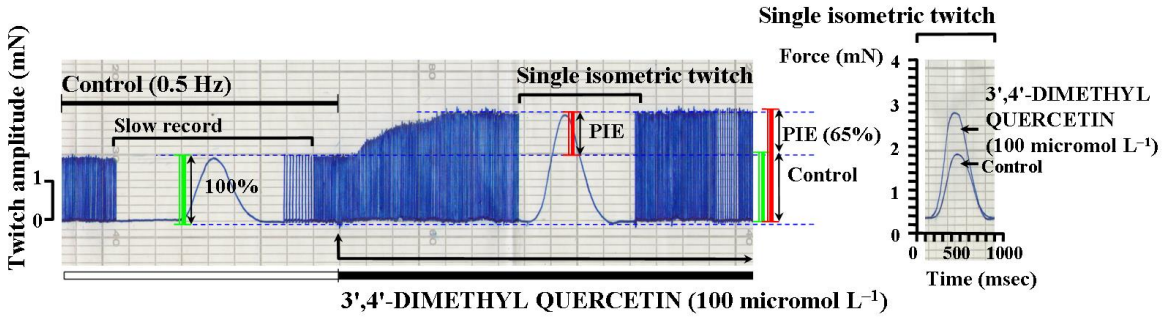

Figure 2. Original recordings of positive inotropic effect of 3',4'-dimethyl quercetin on a rat papillary muscle. Stimulation: 0.5 Hz with pulses of amplitude 5 V, and 5 msec (+36±0.5 °C)

The condition where the maximal effected concentration (100 μmol·L⁻¹) of 3',4'-dimethyl quercetin, the isometric developed force of papillary muscle preparation was increased from 2.345±0.1 mN (the control basal value) to 3.923±0.2 mN or 67.31±5.2% in comparison with the control group ($P < 0.05$; n=4). In these conditions, the EC_{50} value (the values of concentration for 50% of the maximal effect) of 3',4'-dimethyl quercetin was 13.8 μmol·L⁻¹or pD_2 ($-\log EC_{50}$) = 4.86.

Role of potential-dependent $Ca^{2+}{}_L$-channels and adrenergic receptors in the inotropic effects of 3',4'-dimethyl quercetin. Studies have shown that in incubation to inhibit potential-dependent Ca^{2+}-channel – nifedipine (0.01 μmol·L⁻¹) the positive effect of 3',4'-dimethyl quercetin decreases to 21.4±3.6% of control values (Figure 3).

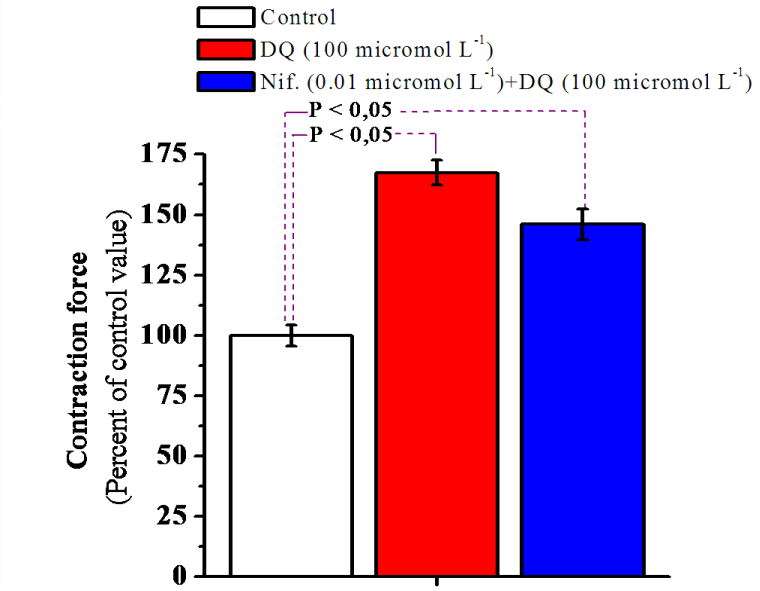

Figure 3. Comparison of the inotropic effects of 3',4'-dimethyl quercetin and nifedipine on the contraction force of extracted rat papillary muscle. Stimulation: 0.5 Hz, 5 V, 5 msec, +36±0.5 °C, resting tension = 10 mN. Data shown for 3',4'-dimethyl quercetin (DQ) (100 μmol·L⁻¹) and nifedipine (Nif.) (0.01 μmol·L⁻¹) were presented as means ± of 4 experiments. $P < 0.05$ indicates value compared to control

The results of experiment demonstrate that the positive inotropic effect of 3',4'-dimethyl quercetin on papillary muscle is not completely connected with potential-dependent $Ca^{2+}{}_L$-channel cardiomyocytes.

It is known that phentolamine and prazosin are inhibitors of α-adrenergic receptors and they reduce the positive inotropic effect of α-adrenergic receptors agonists at the papillary muscle which was extracted under higher concentrations [13]. In the experiment, the inhibitor of α-adrenergic

receptors – phentolamine (5 μmol·L⁻¹) and prazosin (10 μmol·L⁻¹) did not show significant effect on the positive inotropic effect of 3',4'-dimethyl quercetin. Moreover, preincubation of the papillary muscle with phentolamine (5 μmol·L⁻¹) and prazosin (10 μmol·L⁻¹) did not influence on the positive inotropic effect of 3',4'-dimethyl quercetine (100 μmol·L⁻¹) (data is not given). The positive inotropic effect of 3',4'-dimethyl quercetin was not dependent on activation of α-adrenergic receptors.

The positive inotropic effect of 3',4'-dimethyl quercetin was considerably decreased at blocking stage of β-adrenergic receptor with (±)-propranolol hydrochloride (10 μmol·L⁻¹). Figure 4 illustrates concentration – response curves for the positive inotropic effect of 3',4'-dimethyl quercetin and (±)-propranolol hydrochloride (10 μmol·L⁻¹) on rat papillary muscles at 0.5 Hz stimulation (Figure 4).

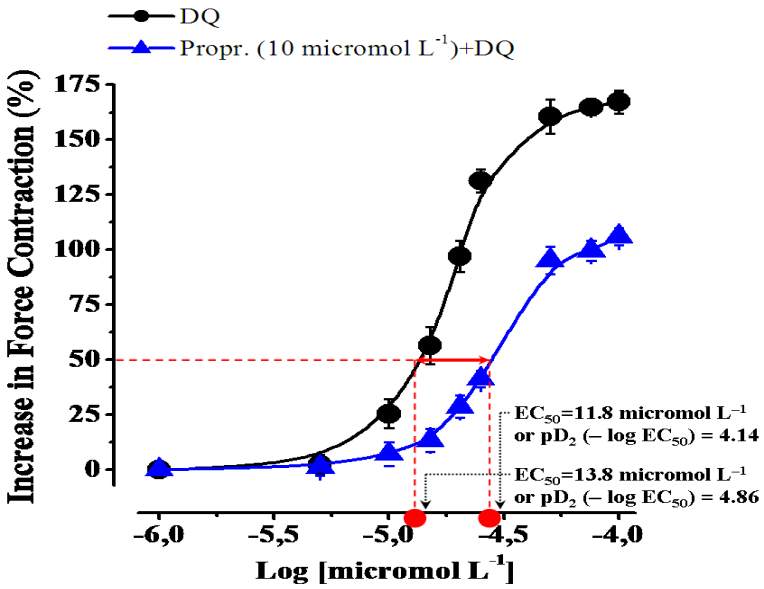

Figure 4. Concentration–response curves for the positive inotropic effects of 3',4'-dimethyl quercetin and (±)-propranolol hydrochloride. Propranolol hydrochloride (Propr) (10 μmol·L⁻¹) was added 10 min before the addition of 3',4'-dimethyl quercetin (100 μmol·L⁻¹) to the incubation. Results are given as means ±S.E.M., n = 4. Stumulation: 0.5 Hz, 5 V, 5 msec, +36±0.5 °C, resting tension = 10 mN

In addition, the positive inotropic effect of 3',4'-dimethyl quercetin was almost completely disappeared in the presence of inhibitor of potential-dependent Ca^{2+}_L-channel – nifedipine (0.01 μmol·L⁻¹) and inhibitor β-adrenoreceptor – (±)-propranolol hydrochloride (10 μmol·L⁻¹) under incubation conditions (Figure 5).

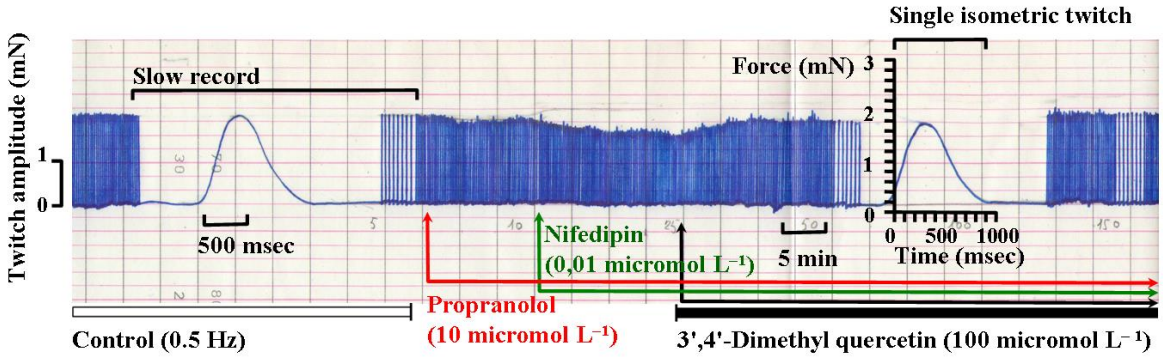

Figure 5. Original recordings for the influence of nifedipine (0.01 μmol·L⁻¹) and (±)-propranolol hydrochloride (10 μmol·L⁻¹) on positive inotropic effect of 3',4'-dimethyl quercetin in a rat papillary muscle. Stumulation: 0.5 Hz with pulses of 5 V, and 5 msec (+36±0.5 °C)

In experiments, in order to analyze the parameters of contraction and relaxation of the papillary muscles, the maximum amplitude force of isometric contraction, the maximum velocity of force development ($+dF/dt_{max}$) and the maximum velocity of relaxation ($-dF/dt_{max}$) were calculated (Figure 6).

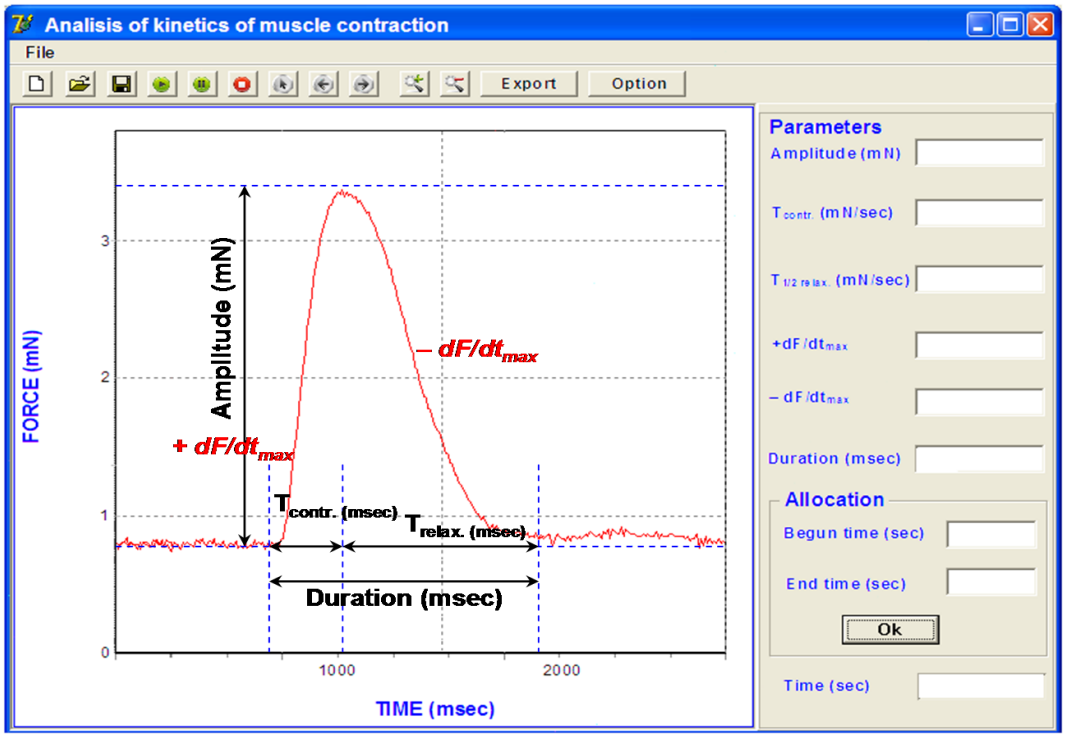

Figure 6. The parameters of single isometric contraction of the extracted rat papillary muscle of control group. This experimental report was obtained by means of the special software "Analysis of kinetics of muscle contraction", created with cooperation of Tashkent University of Information Technology [11]

The mechanical velocity parameters of papillary muscles contractile force are expressed in mN/msec. The investigation showed that 3',4'-dimethyl quercetin dose-dependent (10–100 μmol·L^{-1}) increased the maximum velocity of force development ($+dF/dt_{max}$), and the maximum velocity of relaxation ($-dF/dt_{max}$) (*lusitropic effect*) of papillary muscles (Table).

Table 1: Effect of different concentrations of 3',4'-dimethyl quercetin on the parameters of isometric contraction of the rat isolated papillary muscle of experimental and control group

Concentration of 3',4'-dimethyl quercetin (μmol·L^{-1})	**Parameters of contraction and relaxation kinetics of rat papillary muscle**			
	$T_{contr.}$ (msec)	$T_{1/2\ relax.}$ (msec)	$+dF/dt_{max}$ (mN/msec)	$-dF/dt_{max}$ (mN/msec)
Control	278.46	435.96	0.0084	0.0026
10	223.51	397.58	0.0097	0.0028
25	210.27	376.74	0.0104	0.0031
50	187.32	364.21	0.0109	0.0036
75	168.43	356.31*	0.0111*	0.0043
100	121.96*	321.02	0.0113*	0.0048*

Notes: $T_{contr.}$ – time from peak tension; $T_{1/2\ relax.}$ – time from to 50% relaxation; $+dF/dt_{max}$ – the maximum velocity of force development; $-dF/dt_{max}$ – the maximum velocity of relaxation; Parameters in control group were registered during the perfusion with physiological Krebs–Henseleit solution (pH = 7.4), and stimulation: 0.5 Hz with pulses of 5 V, and 5 msec (+36±0.5 °C). * – $p<0.05$ as compared to control group ($n = 5$).

It was observed from the experiments that 3',4'-dimethyl quercetin (100 μmol·L^{-1}) increased isometric contraction amplitude of the rat isolated papillary muscle from 2.345 mN up to 3.923 mN or 67.31% in comparison with the control. And also, at action this flavonoid (100 μmol·L^{-1}) decreased the duration of single isometric contraction for 34% in comparison with the control or from 1150 msec up to 764 msec (time from peak tension and to 50% relaxation, respectively). Thus, the parameter $+dF/dt_{max}$ increased to 34.1±3.2% in comparison with a group of the control with influence of that 3',4'-dimethyl quercetin (100 μmol·L^{-1}). Furthermore, the maximum velocity of relaxation $-dF/dt_{max}$ was considerable increased to 46.2±4.1% in comparison with a group of the control.

It is identified that the maximum velocity of force development ($+dF/dt_{max}$) and the maximum velocity of relaxation ($-dF/dt_{max}$) of the rat papillary muscles were significantly high in the experimental group than in the control group ($P < 0.05$).

Discussion

In many research studies, the cardiovascular effects of bioflavonoids were investigated. For example, in condition *in vitro*, it is shown that some flavonoids possess positive inotropic and lusitropic effects on isolated heart of experimental animals. Thus, authors of these works have supposed that these effects of flavonoids are connected with inhibition of phosphodiesterase (3'-5'-cAMP-phosphodiesterase) enzyme [14, 15].

It is shown that many of developed positive inotropic agents are phosphodiesterase inhibitors. The mechanism of the positive inotropic effect of phosphodiesterase inhibitors, an inhibition of the degradation of cAMP are explained with an increase in $[cAMP]_{in}$. This process leads to an increase in the I_{CaL} inward current during the AP, and leads to an increase in Ca^{2+} release from sarcoplasmic reticulum (SR), to an increase in the $[Ca^{2+}]_{in}$ and, therefore, to a positive inotropic effect [16].

Other investigations showed that flavonoids demonstrate a positive inotropic effect, through an increase in cAMP, which increases $[Ca^{2+}]_{in}$ [17–21].

It is known that, increasing concentration of $[cAMP]_{in}$ activates protein kinase A, which phosphorylates the L-type Ca^{2+}-channel, troponin I, and causes an increase of $[cAMP]_{in}$ and subsequently phosphorylation of contraction-controlling proteins, including $Ca^{2+}{}_{L}$-channels occured, and the amplitude force of contraction papillary muscles increased as well. Phosphorylation of these $Ca^{2+}{}_{L}$-channels promotes Ca^{2+} influx that triggers the release of Ca^{2+} from the RyRs of SR and $[Ca^{2+}]_{in}$ transient finally activates the contraction system of myocardium [22].

In some researches, only negative inotropic effects of flavonoids on contractility of cardiac muscles were shown. For example, it is identified that the fraction of flavonoids which extracted from plant *C. lyratiloba* decreases the amplitude force of rat papillary muscles [23].

And also, there are other impact mechanisms of flavonoids. For instance, at the department of Biophysics, National University of Uzbekistan (Tashkent, Uzbekistan), Umarova et al. (1998) it was investigated that flavonoids considerably inhibited the activity of the Na^{+},K^{+}-ATPase [24]. In addition to this Schüssler et al. (1995) determined that a positive inotropic effect of some flavonoids is connected with activation of adrenergic receptors in cardiomyocytes [14].

Thus, the obtained data in this research (Figure 4; 5) and the analysis of data from the different researches published on the basis of its results, it can be assumed that the positive inotropic effect of 3',4'-dimethyl quercetin happens to be based on cAMP-dependent mechanism (Figure 7).

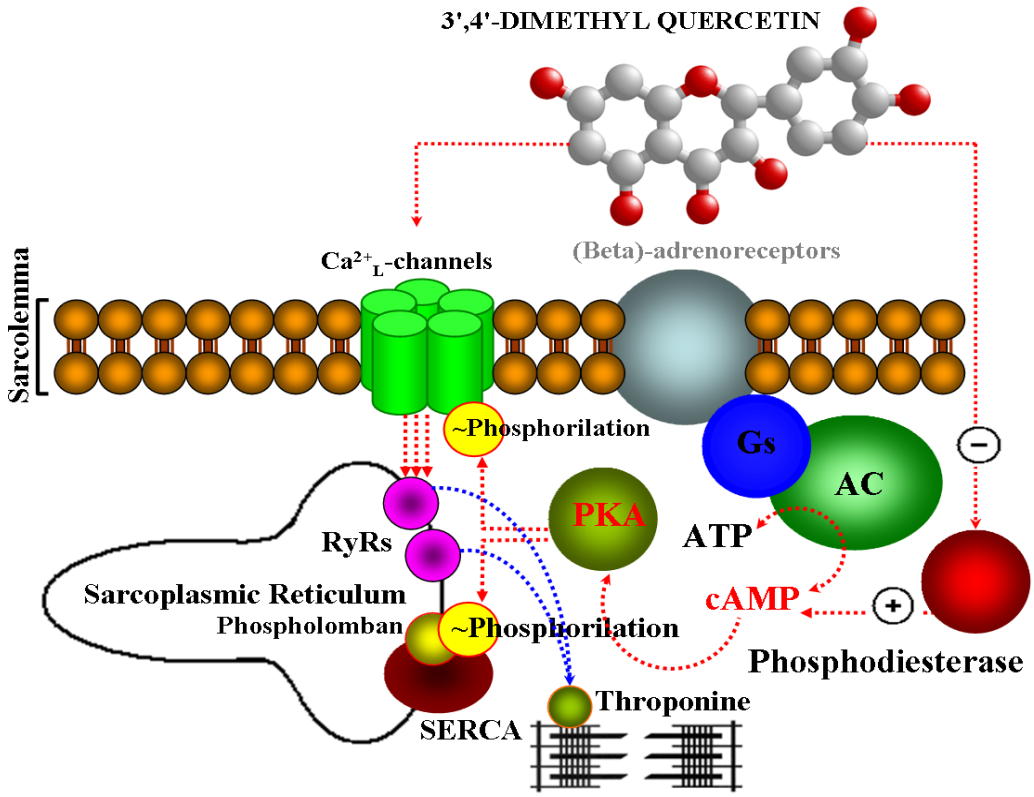

Figure 7. Hypothetical inpact mechanisms of inotropic effect of 3',4'-dimethyl quercetin on the rat myocardium. RyRs – Ryanodine Receptors or Sarcoplasmic reticulum Ca^{2+}–channels; SERCA – The Sarcoplasmic reticulum Ca^{2+}–ATPase; ATP – Adenosintriphosphat; G_s – Guanine nucleotide-binding proteins; AC – Adenylate cyclase; cAMP – Cyclic Adenosine 3',5'–monophosphate; PKA – proteine kinase A

In the experiment, it is shown that 3',4'-dimethyl quercetin dose-dependent (10–100 μmol·L^{-1}) increased in the maximum velocity of force development ($+dF/dt_{max}$), and the maximum velocity of relaxation ($-dF/dt_{max}$) of papillary muscles (*lusitropic effect*) (Table). This means that the contraction and relaxation kinetics are critical determinants of cardiac performance and currently the mechanism, regulation of myocardial contraction and relaxation kinetics are almost completely understood [25, 26].

According to Korotkich et al. (2006), an extract (the basic part consists from flavonoids) from plant *P. frutescens* (L) under *in vitro* condition shows positive inotropic and lusitropic effects on the rabbit myocardium. Also, the scientists supposed that such effects of *P. frutescens* (L.) extract can be connected with the increase of inward Ca^{2+} ions through $Ca^{2+}{}_L$-channels in the sarcolemmal and the increase of $[Ca^{2+}]_{SR}$. Moreover, the lusitropic effects of extract *P. frutescens* (L.) is explained with increase in $[cAMP]_{in}$ or inhibition of activity of phosphodiesterase enzyme. Through the rising process of concentration $[cAMP]_{in,}$ PKA activation occurs and $[Ca^{2+}]_{in}$ increases through the sarcolemmal $Ca^{2+}{}_L$-channels, "Ca^{2+} induced Ca^{2+} release" (CICR) from SR increases, and also at phosphorylation of phospholamban increases activation Ca^{2+}-ATPase of SR, parameter $-dF/dt_{max}$ increases as well [27].

Conclusion

In conclusion, the present study demonstrates that the positive inotropic effects of 3',4'-dimethyl quercetin in the rat papillary muscles can be mediated by increase in $[cAMP]_{in}$ which increase $[Ca^{2+}]_{in}$. And also, lusitropic effect of 3',4'-dimethyl quercetin increases in cAMP and inhibition of phosphodiesterase enzyme activates proteine kinase A, which phosphorylates regulator protein activity Ca^{2+}-ATPase – phospholamban and sarcoplasmic reticulum Ca^{2+}-channel (RyRs).

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УДК 615.2

Инотропное действие 3'4'-диметилкверцетина на сократительную активность папиллярной мышцы крысы

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Аннотация. Целью настоящего исследования явилось изучение инотропного действия 3'4'-диметилкверцетина на функциональную активность миокарда крыс. Регистрацию изометрической сила проводили с помощью механотрона (F30/Model D-79232; Hugo Sachs Elektronik, March-Hugstetten, Германия). Установлено, что 3'4'-диметилкверцетин (100 – 200 мкМ) вызывает положительный инотропный эффект. При этом, в присутствие блокатора потенциалзависимых $Ca^{2+}{}_L$-каналов (нифедипин) и блокатора β-адреноблокатора (пропранолол) положительный инотропный эффект 3'4'-диметил кверцетина почти полностью уменьшается. А также, 3'4'-диметилкверцетин дозозависимо увеличивает максимальную скорость развития силы и максимальную скорость расслабления папиллярной мыщцы.

Таким образом, полученные данные позволяют предположить, что положительный инотропный эффект 3'4'-диметилкверцетина может быть связан с активацией β-адренорецепторов, при этом увеличивается концентрация цАМФ и следовательно увеличивается $[Ca^{2+}]_{in}$ в кардиомиоцитах. А также, луситропный эффект 3'4'-диметилкверцетина может быть связан с увеличением цАМФ; блокированием фермента фосфодиэстеразы; активацией протеинкиназы А и фосфорилированием регуляторного белка Ca^{2+}-АТФазы – фосфоламбана и фосфорилированием RyR.

Ключевые слова: папиллярная мышца, 3',4'-диметил кверцетин.

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A Case Series: Outcome of Endoscopic Electrocautery in the Management of Branchial Fistula

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Abstract

Objective: This is a series of five cases of branchial anomalies which were diagnosed and treated in a span of six years in the Department of Otorhinolaryngology, Head & Neck Surgery, Universiti Kebangsaan Malaysia Medical Centre (UKMMC). The main objective of this article is to highlight the use of endoscopic electrocautery in the management of branchial fistula. Case report: five cases were reported of the age group between 11 months old to 16 years who presented with an intermittent mucoid discharge from an external opening in the neck since birth and three cases were on the left side and the other two cases were bilateral fistula. Direct laryngoscopy under general anaesthesia was done as part of diagnostic and therapeutic management for the patients. Conclusion: Endoscopic electrocautery is a safe method and appears to be an effective alternative to open excision for branchial fistula.

Keywords: branchial fistula, endoscopic, electrocautery, branchial sinus.

Introduction

Branchial sinus, fistula or cysts are manifestations of a failure of branchial cleft to involute. Branchial anomalies most commonly present during infancy and childhood as cutaneous sinus, cyst or an abscess. Anomalies of the second branchial fistula account for 90 % of the developmental abnormalities of the branchial apparatus. The reported prevalence of third and fourth arch anomalies accounts to 1–4 % of all branchial anomalies. [1]

Branchial fistula usually presents in childhood discharge and defect along the anterior border of sternocleidomastoid muscle at the junction of middle and lower l/3rd of neck. Traditionally, management consist of treatment of acute infection, followed by surgical excision of the tract and obliteration of the opening. However, recently, it has been suggested that obliteration of the sinus tract alone using laser, chemo or electrocautery is a viable alternative to open surgery.

Objective

This is a series of five cases of branchial fistula which were diagnosed and treated in a span of six years (2009-2015) in the Department of Otorhinolaryngology, Head & Neck Surgery, Universiti Kebangsaan Malaysia Medical Centre (UKMMC). The main objective is to highlight the use of electrocautery obliteration for the management of branchial fistula.

Materials & Methodology

Retrospective case note review of all children that received treatment of branchial fistula in the past six years at UKMMC. Patient's demographics, presenting symptoms, investigations and surgical management and operative outcome were analysed.

Results

There were five cases managed at the Department of ORL-HNS UKMMC and diagnosed with second and third branchial fistulae during the 6 years. They were in the age group between five months and 16 years with male: female ratio being 2:3. Three cases were on the left side and the two cases had bilateral fistula. All of these patients presented with history of small discharging opening in the lower part of the neck since birth. There were symptoms of intermittent, yellowish white discharge from the opening. The discharge from the sinus opening was associated with fever in three of the cases. On examination, a small opening was seen on neck along the anterior border of the sternocleidomastoid at junction of middle and lower third with scanty mucous discharge especially on digital pressure. Examination of the oral cavity did not reveal any visible opening in the pharynx or palatine tonsil. After necessary routine investigations, all patients underwent examination and direct laryngoscopy under general anaesthesia. Once patient was under general anasthesia, microsuspension was then performed with a Lindholm laryngoscope. The opening of into the pyriform sinus was identified and visualised when methylene blue was injected via the cutaneous opening. Spillage were seen at left pyriform fossa in three of the cases and in one case the internal opening was located at left superior pole of palatine tonsil and the other case, which was bilateral, the internal opening were seen at right superior and left middle pole of palatine tonsil. An electrocautery (diathermy) was then used, where it was placed into the sinus tract to its base through its internal opening and stimulated until the surrounding tissue began to blanch and then stimulated until the sinus opening was cauterized. Patient was then reversed from general anaesthesia and nursed in ward for 24 hours before discharged. Three of the patients presented with recurrence. Two of the patients underwent excision of the sinus tract that was carried out under general anaesthesia. Aim of surgery was to perform complete excision of fistulous tract which became a shorter sinus tract after initial cauterization and this ease the surgery. One of the patients with recurrence underwent re-diathermy over the internal opening of the fistula. All three patients are well, and had no recurrence after six months post operation.

Table 1: Demographic and history of presenting illness of the patients

Num	Age	Sex	Age during first symptom	Symptoms	Previous I&D	Imaging
Case 1	5 months old	F	5 months old	Mucoid discharge from sinus opening	Nil	MRI – Left sinus opening from the neck, open at the anterior pillar – anterior to left tonsil at level of tip of epiglottis Right sinus opening tract up to a blind sac without any obvious opening

Case 2	8 years old	F	6 years old	Neck swelling, fever, pus discharge from sinus opening	Yes – 6 times	CT scan – Infrahyoid midline and left paramedian abscess collection extending into left SCM*
Case 3	4 years old	M	2 years old	Neck swelling, fever, pus discharge from sinus opening	Yes – 4 times	Ultrasound – Heterogeneous collection at left anterior triangle with air filled tract from overlying skin to left side of trachea – no communications with trachea
Case 4	11 months old	F	11 months old	Mucoid discharge from sinus opening	Nil	MRI – Right anterior neck opening, ends at lat pharyngeal wall below palatine tonsil. Left anterior neck opening directed superomedially and opens posterior to palatine tonsil
Case 5	16 years old	M	6 years old	Neck swelling, fever, pus discharge from sinus opening	Yes – 8 times	CT scan – Sinus tract leading to area deep to left SCM. Barium swallow – opening seen at left pyriform fossa

*SCM – Sternocleidomastoid; F-Female; M-male

Table 2: Surgical management and follow up

Case	DL scopy and Intra op findings	Treatment	Recurrence	Treatment/Outcome
Case 1	Left branchial fistula tract from anterolateral neck between junction of superior 1/3 and inferior 2/3 SCM* to superior pole of left tonsillar fossa No internal opening over right side	Internal opening diathermised	Yes	Excision of left branchial fistula. Well and no recurrence until now.
Case 2	Blind tract noted at left pyriform fossa. Right pyriform normal	Tract and internal opening diathermised	Yes	Re-diathermised Patient is well until now
Case 3	Fistula seen at anterior part of pyriform fossa	Internal opening diathermised	No	Well
Case 4	Internal opening at right superior pole of palatine tonsil and left interior opening at middle part of palatine tonsil	Internal opening diathermised	Yes	Excision of right branchial fistula and re-diathermised the left internal opening. Patient is well until now.
Case 5	Opening of fistula seen at left pyriform fossa near the anterior aspect	Internal opening diathermised	No	Well until now

*SCM - Sternocleidomastoid

Discussion

Branchial fistulas and cysts, involving soft tissues of the head and neck, are uncommon anomalies of embryonic development. It may occur in any age group, but the first and second decades of life are the most common. Branchial cysts have been found to be more prevalent (80.8 %) than branchial fistulas (19.2 %) [1]. The presence of bilateral branchial anomalies has been reported in 1 % to 30 % of cases. [1] In this case series, we have encountered two cases of

bilateral branchial anomalies which were confirmed by MRI and examination under anaesthesia, one was confirmed by barium swallow and the others were confirmed by CT scan.

Second branchial arch and pouch anomalies are common anomalies of branchial apparatus, account for 90 % of the developmental abnormalities of the branchial apparatus. A study done in UKMMC between 1999-2009, 12 patients were diagnosed with branchial anomalies, in which 10 patients had second branchial cyst anomalies, 1 had third branchial fistula and 1 had bilateral branchial lesion [2]. Second arch anomalies may take several forms. There may be only a simple sinus opening that extends up the neck with a variable distance. Branchial fistulas commonly present clinically with persistent mucoid discharge from a skin opening in the neck as can be seen in the cases described in this case series.

Third and fourth branchial fistulae, also known as pyriform sinus fistulae, are epithelialized tracts connecting the skin of the neck to the foregut. Persistence of this duct results in a sinus tract that communicates with the pyriform fossa, representing persistence of both branchial cleft and corresponding pouch [1,3].

As with branchial anomalies of the second pharyngeal pouch, the external opening of both third and fourth pouch remnants arises at the same location in the skin overlying the anterior border of the sternocleidomastoid muscle, which is the location of the embryologic cervical sinus.

It is difficult to differentiate between fourth from third branchial arch anomalies as there are some overlapping features, and precise identification of anatomic relationships at the time of diagnosis and treatment is not always possible [1]. The differentiation between the two conditions lies in determining the relationship of the sinus to the superior laryngeal nerve, which can only be identified with surgical exploration. If the sinus passes below the superior laryngeal nerve, a fourth branchial pouch sinus is suggested, whereas if the sinus passes above the superior laryngeal nerve, a third branchial pouch sinus is suggested.

A combination of ultrasound, computed tomography (CT) with or without oral contrast, barium swallow study looking for the sinus tract, thyroid scan, or magnetic resonance imaging (MRI) may aid in the diagnosis [5]. However, direct laryngoscopy often allows visualization of the fistulous opening in the pyriform fossa and can be performed during acute episodes [6]. In our centre, these five cases, CT scan with oral contrast was performed, with combination of barium swallow study in two of the cases. Direct laryngoscopy was done in all cases as part of confirmation of diagnosis and also treatment. The cases that required barium swallow were referred after multiple incision and drainage for recurrent neck abscess. Fistulogram was difficult to delineate any tract due to fibrosis and barium swallow was able to show the tract prior to direct laryngoscopy.

Treatment should be preceded by the administration of appropriate antibiotics during acute infection and other methods of treatment should commence once inflammation has subsided [8]. Curative procedure should only be attempted in the absence of acute infection [1, 3 and 5].

Surgery is the definitive treatment but usually not indicated if the fistula is asymptomatic. However, most cases patients are symptomatic and the surgical excision is carried out to avoid the risk of recurrent infection and also for cosmetic reasons.

Several surgical approaches have been described for the management of branchial fistula. In general, a wide cervicotomy (hockey stick) incision remains the method of choice for excision of lesions of the second branchial clefts as it allows for adequate exposure of neck structures for accurate dissection. Other methods described include the stepladder approach and the stripping method. The skin incision in the stepladder approach is less extensive than the wide cervicotomy incision. Complications of the surgery include recurrence, which could be 3 % in fresh cases to up to 20 % in second surgical attempts. Other complications include secondary infection, injury to facial, hypoglossal, glossopharyngeal, spinal accessory nerves, injury to internal jugular vein, scarring and hematoma formation [11].

However, recently a less invasive treatment has evolved, namely, endoscopic cauterization limited to the sinus tract orifice [5, 8]. A number of endoscopic techniques have been reported in the literature. Verret et al. have described the introduction of a balloon catheter for the dilation of the sinus tract orifice to allow endoscopic cauterization. Electrocautery was also used by Jordan et al. with the aid of a diathermy probe. Sayadi et al. used a low-power diode laser, while two other groups used chemical cauterization with trichloroacetic acid. Kim et al. and Pereira and Smith performed chemical cauterization by introducing a stick of silver nitrate for 3-4 s into the sinus

tract and at the sinus tract orifice [6].

Endoscopic treatment represents a minimally invasive technique using cauterization to obliterate the internal opening of a pyriform sinus tract during a quiescent period. It can be done as a definitive management or along with the surgical excision of the branchial anomaly [4, 5 and 6].

Electrocauterization has been used as one of the treatment modalities for branchial fistula. Possible advantages of this technique over open neck surgery include a lower complication rate with similar rates of recurrence as open neck surgery [3].

In our case report we present five cases of branchial cleft sinus anomaly presented as recurrent neck abscess which two of the cases we successfully managed by a single endoscopic cauterization of sinus tract. In one of the cases there was recurrence after the first cauterization, a repeated cauterization done. All 3 patients are symptoms free for more than 6 months period of follow up.

Conclusion

When compared to other surgical management, this minimally invasive technique offers several advantages over the open procedure. It allows less scarring, less morbidity, minimizes risk of injury to the neck structures and earlier hospital discharge. Endoscopic electrocautery is a safe method and appears to be an effective alternative to open excision. We would like to recommend it as an alternative part of the first line treatment for branchial fistula.

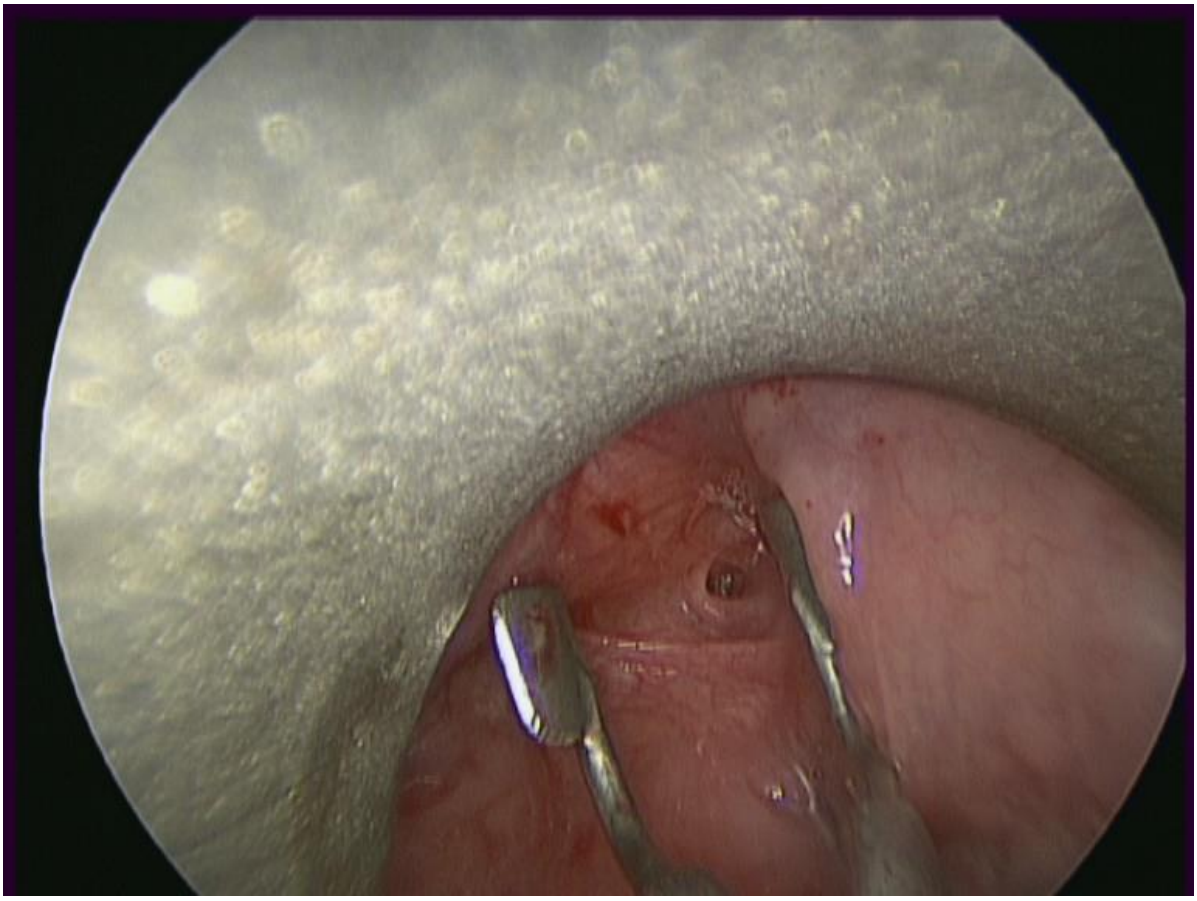

Figure 1: Internal opening seen at the left pyriform fossa. Laryngeal spreader was used to aid visualization.

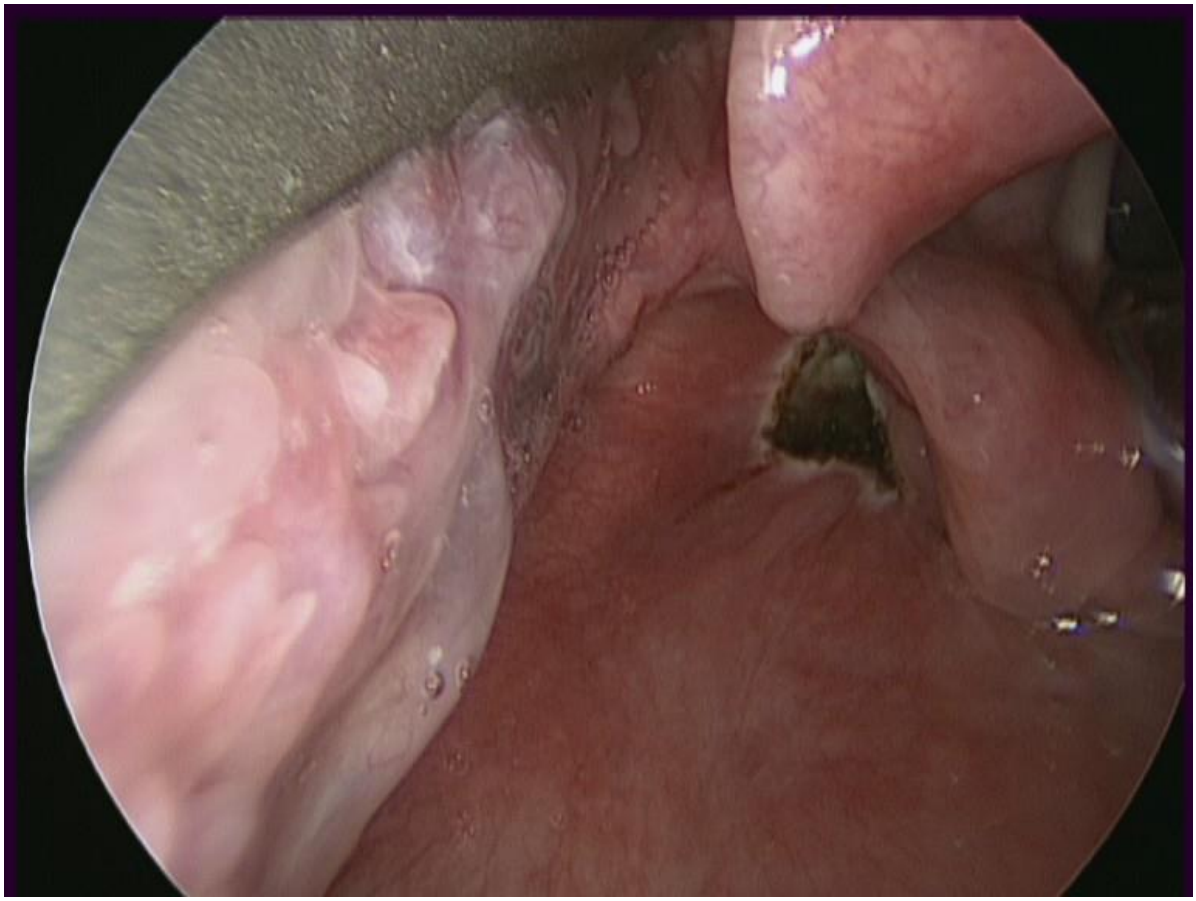

Figure 2: Internal opening over left pyriform fossa after diathermised

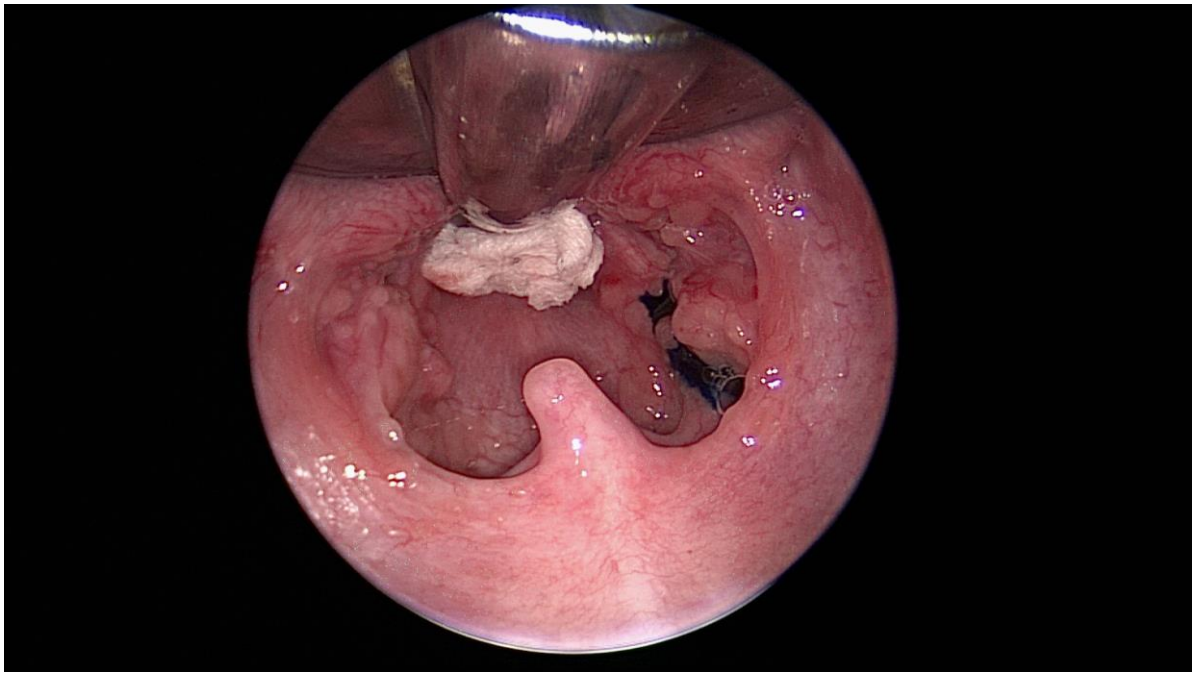

Figure 3: Methylline blue dye seen spilling out from the internal opening at the left tonsillar fossa.

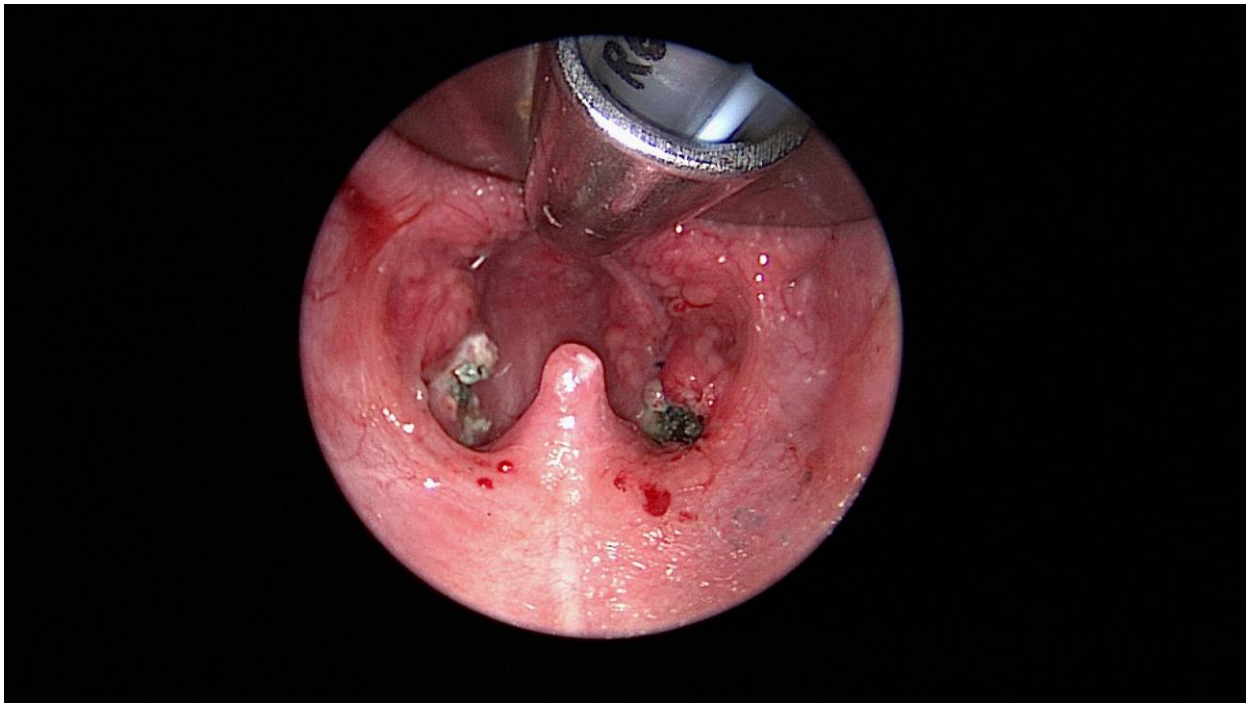

Figure 4: Internal opening over bilateral tonsillar fossa after being diathermised.

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