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Studying of some proteins levels and enzymes activities in the amniotic fluid for pregnant associated with premature birth.

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Highlights

- **This study evaluates the apical leakage and the adaptability of two silicone-based root canal sealers.**
- **Shed the light on the importance of sealing ability of root canal filling material.**
- **Clearing technique and horizontal technique to evaluate the adaptability and presence of void in root canal filling.**
- **The tested silicon-based root canal sealers could not completely prevent dye penetration.**

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ABSTRACT

The research includes studying of some proteins and enzymes levels in amniotic fluid for pregnant associated with premature birth, which includes C-reactive protein (CRP), alpha-fetoprotein (AFP), cystatin C, aminoacylase-1(ACY1), Lactate-dehydrogenase (LDH), aspartate aminotransferase (AST) and alanine aminotransferase (ALT). The study was done in Mosul city on (75) women that had hypertension, and (78) women who healthy pregnancies as a control group, the age of pregnant with hypertension and control group ranged from (16-45) year, then specimens were divided into three age groups: The first group (16-25) year, the second group (26-35) year and the third group (36-45) year.

The results showed that there was a significant increase in the levels of proteins for CRP and AFP for all studied groups compared with a control group. On the other hand, the results of measurements of enzymes in the amniotic fluid indicated that there was a significant decrease in the activity of the ACY1 and a significant increase of enzymes: LDH, AST, and ALT in the amniotic fluid for pregnant associated with premature birth compared with the control group.

High levels of CRP, cystin C, and AFP proteins, can be an indicator of diseases that accompany pregnant women, especially pregnant women with preterm birth. There are clear negative effects about the enzyme variants used to assess liver function in the amniotic fluid of preterm pregnant patients.

1. Introduction

Premature birth can be defined as the birth that occurs before the 37th week of pregnancy and accounts for 10% of births (Martin *et al*., 2015). It is considered one of the most important complications of pregnancy and it is responsible for exposing the newborn to various health risks. In addition, 70% of deaths of births are produced from premature birth, and it has been observed that rupture of early fetal membranes is one of the most important causes of premature birth, which represents 40-30% (Ryan and Black, 2015). The occurrence of premature birth is associated with low birth weight, due to the incomplete maturity of the fetus's organs such as the lung, liver, and kidneys, as the short duration of pregnancy and the low birth weight (Less than 2500 grams) leads to the newborn being exposed to many health risks such as heart and kidney diseases, respiratory diseases, immune diseases, hypertension, growth problems and may in some cases lead to the death of the newborn (Reddy, 2015).

Factors contributing to the occurrence of premature birth include complications of pregnancy such as urinary tract infection, placental abruption, blood clotting, chronic diseases such as high pressure and diabetes (Ryan and Black 2015), as well as an increase or decrease in the volume of amniotic fluid, and when the pregnant woman is less than 18 year and older than 40 years, in addition to poor living conditions, reduced health care, anemia, malnutrition, exposure to various environmental pollutants from smoking, drugs, radiation, heavy metals, and chemicals, as well as psychological and physical stress (Kumar *et al*., 2015).

The C-reactive protein (CRP) has been observed that there is an increase in the level of CRP during pregnancy, especially in the 15th week of pregnancy, and its level increases more at birth, in addition to that, the level of CRP increases an indication of the occurrence of pregnancy complications and increases the risk of premature labor, spontaneous abortion, placental abruption, and lack of blood flow to the fetus, which results in many health problems for the fetus (Shah and Baxi, 2016).

The alpha-fetoprotein (AFP) level rises in the amniotic fluid from week 14 of pregnancy and reaches its highest level at 22 weeks, then its level decreases before birth (Bartkute *et al*., 2017), so its high level is considered an indication of the presence of fetal disorders. As its level in the amniotic fluid may be elevated in the case of fetal malformations such as neural tube defect and spina bifida (Başbuğ *et al*., 2017). As for cystatin C, its concentration in the blood is related to the integrity of renal function (Tomotaki *et al*., 2017) and it increases in the weeks between 20-30 weeks of pregnancy (Sawada *et al*., 2019), as cystatin C is a sensitive indicator to know the extent of fetal disease. It is used to diagnose and monitor

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fetal kidneys and the occurrence of malformations (Tomotaki *et al.*, 2017).

Aminoacylase-1 (ACY1) [EC 3.5.1.14] works to reduce the effects of toxic substances resulting from oxidation processes, and it has a role in the pharmaceutical industries and in the production of free amino acids that are used in building protein (Ivanov *et al.*, 2014) as well as in the process of regulating the urea cycle by acting as an allosteric regulator using carbamoyl phosphate synthetase and then get rid of the excess ammonia (NH3) produced in the body by converting it into urea, and it also has a regulatory role during starvation and malnutrition during metabolic processes (Berg *et al.*, 2012). Van coster *et al.* (2005) showed a decrease in ACY1 activity in pregnant with congenital anomalies and encephalopathy, and Tylki-Szymanska *et al.* (2010) showed that there is a relationship between enzyme deficiency and autism, as the N-acetyl-glutamate compound has a biological role In learning, perception and memory, changing its level causes many brain functions (Tylki-Szymanska *et al.*, 2010).

The LDH enzyme was observed to increase during pregnancy and is associated with the risk of pregnancy complications such as placental abruption, kidney failure, spontaneous abortion and premature birth, and it also exposes the fetus at risk of stunted growth (Andrews and Patel, 2016). Besides, it has been observed that there is an increase in the level of the ALT during pregnancy associated with acute and chronic liver disease, cholangitis and jaundice, and its level also increases in cases of cirrhosis and liver cancer (Westbrook *et al.*, 2016), and it was also noted that An increase in enzyme activity (AST and ALT) during pregnancy also occurs in cases of hypertension, as well as in preeclampsia and Hellp syndrome (which is one of the complications of pregnancy characterized by hemolysis) and when taking steroid medications (Mao and Chen, 2016). This study aims to estimate the levels of proteins and enzymes, to assess the extent of this reflection on the pregnant mother with premature birth.

2. Materials and methods

Amniotic fluid samples were collected from pregnant during childbirth from Al-Khansa Teaching Hospital in the city of Mosul and under the supervision of specialized doctors in the maternity hall, for the period between October 2017 and the end of February 2018, and the residents of different locations within the city of Mosul, and after recording the required information, collect (78) women of amniotic fluid from pregnant without diseases, and (95) women were collected from pregnant with hypertension associated with pregnancy and ages ranged between (16-45) years for both groups.

Amniotic fluid samples were taken from pregnant before birth through the transvaginal and using a special instrument called the Sims speculum. The liquid was withdrawn quietly from the inside of the amniotic membrane by a sterile syringe and the liquid was transferred directly to the laboratory, and the liquid was taken in a volume of (10) ml and then it was performed centrifugation at a speed of (3000) xg for (20) minutes to obtain the clear liquid, and some tests were conducted directly, and the remainder of the liquid was preserved by freezing at a temperature of (−20) °C until the rest of the tests were carried out (Soydin *et al.*, 2013).

2.1 Methods used to measure the proteins and enzymes specified in the research

Determine the level of the C-reactive protein in the amniotic fluid using the ready-made analyzes produced by BioKit- Sa (Spanish company), which contain ready-made solutions and reagents based on the use of the Latex Agglutination reaction, and the level of alpha-fetoprotein using Boditech from the Korean company, which depends on the use of the Fluorescence immunoassay (FIA) method, which depends on the binding of the antibody to a fluorescent substance in the antigen buffer in the sample forming a complex Antigen-Antibody (Wespic, 1989).

The level of cystatin C was estimated using Veda lab kit from the French company, which depends on the binding of the monoclonal dye to the antibodies in the polyclonal solid phase to determine the amount of cystatin C present in the sample that interacts with it to give a red-purple color. The color intensity is measured in the VEDA Lab (Inker, 2012).

The activity of the enzyme aminoacylase-1 was estimated according to the method used by Peterson (1983), which depends on the principle of hydrolysis of aliphatic amino acids that contain acetyl such as N-acetyl methionine to give acetic acid and methionine, and that the level of methionine is estimated concentrated using the ninhydrin method (Rosen, 1957), by the following:

2.3 The solutions used

1- The buffer solution (Tris-HCl) (50) mmol and pH 7.5.

2- N-acetyl methionine at a concentration of (15) mmol.

3- 15% Trichloro acetic acid (TCA).

4- Nanhydrin solution at a concentration (3%).

5- Sodium cyanide solution (NaCN) at a concentration of (250) micromole.

6-Isopropanol: prepared at a concentration of 50% (V/W).

3. Procedure

1- Add (0.6) ml of the buffer solution in a test tube, and then add (0.1) ml of the amniotic fluid and mix well, which is considered the test solution.

2- Add (0.6) ml of the buffer solution in a test tube, then add (0.1) ml of distilled water and mix well, which is considered a Blank solution.

3- Adding 0.6 ml of the substrate N-acetyl methionine to each of the previous test tubes and then measuring the activity of the enzyme by ninhydrin method, at time zero and after 30 minutes of adding the substrate.

4- Add 0.5 ml of TCA after 30 minutes adding the substrate, then put it in the centrifuge.

5- 0.5 ml of the filtrate is taken and 0.5 ml of ninhydrin solution is added, and placed in a boiling bath for 15 minutes.

6- (1.5) ml of isopropanol is added, then the absorbance intensity is measured at a wavelength of 570 nm, and then the enzyme activity is calculated according to the following:
Enzyme activity (U/L)=difference in absorbance before and after 30 minutes of adding the substrate x 69. As factor 69 represents the product of the molar absorption coefficient of methionine X the volume of reactants X the time required for the reaction to occur.

As for the activity of the enzymes LDH, AST, and ALT, it was estimated using kit type BioLabo from the French company, as it was used by researchers (Klin and Klin, 1972) in estimating the activity LDH, besides the enzymes AST and ALT, determination by the method of researchers (Reitman and Frankel, 1957).

4. Statistical analysis

The SPSS-17 statistical program was used to determine the mean and standard deviation (SD). The t-test was chosen to compare two variables and find the difference between the values that appeared through the P-value (which occurs at P≤0.05), a significant difference (Hinton, 2004).

5. Results and discussion

5.1. Comparison of levels of proteins in the amniotic fluid of pregnant associated with premature birth with a healthy pregnant group

5.1.1. C-reactive protein (CRP)

The results are shown in Table (1) indicate that there is a significant increase in the levels of CRP in the amniotic fluid of pregnant with premature birth compared with the group of healthy

pregnant at P≤0.0001 for all age groups, and these results are consistent with the results of previous studies that showed that there is an increase in the level of CRP in the amniotic fluid of pregnant with premature birth (Keikhaie *et al*., 2018) and another study is showed increased in CRP in the blood of pregnant with premature birth (Deo *et al*., 2016), and that referred to increase the production of inflammatory cytokines in response to bacterial infection and the occurrence of inflammation in the amniotic fluid, which stimulates prostaglandins (Stepan *et al*., 2016).

The level of CRP also increases as a result of rupture of early fetal membranes, causing bacteria and viruses to leak into the amniotic sac, causing placental inflammation and chorioamnionitis, as infections inside the uterus contribute to the occurrence of premature birth by 50-40% as it causes rupture of early fetal membranes, the premature rupture of the membranes (Musilova *et al*., 2017), and this rupture is one of the causes of premature labor for 30% (Stepan *et al*., 2016; Deo *et al*., 2016), and premature birth exposes the fetus to risks of developmental disability, heart disease and death after birth (Silverberg *et al*., 2018).

Table (1)

Protein levels in the amniotic fluid of preterm pregnant com

Measured proteins	Protein levels in the amniotic fluid according to age groups of pregnant (mean ± SD)					
	First age group (16-25) Year		Second age group (26-35) Year		Third age group (36-45) Year	
	Healthy pregnant (N=24)	Pregnant with preterm birth (N=25)	Healthy pregnant (N=30)	Pregnant with preterm birth (N=22)	Healthy pregnant (N=24)	Pregnant with preterm birth (N=18)
CRP (mg/100mL)	4.5 ±0.2	***44.6±7.1	3.5 ±0.3	***46.6 ±6.1	5.0 ±0.45	***58.0±9.6
AFP (ng/mL)	89.3±3.9	***228±5.9	3.8±78.8	***289±2.3	103± 3.4	***332±3.7
Cystatin C (mg/L)	0.8 ±0.14	0.9±0.1	1.2±0.09	1.3±0.06	0.9±0.05	0.9±0.04

*** Significant difference at P ≤ 0.0001. CRP: C-Reactive protein, AFP: Alpha Feto protein.

5.1.2. Alpha Feto Protein (AFP)

The results are shown in Table (1) indicated a significant increase in the levels of alpha-fetoprotein in the amniotic fluid of pregnant with premature birth compared with the group of healthy pregnant at P ≤ 0.0001 for all age groups, and these results are consistent with the results of the previous study that showed that there is an increase in the level of AFP in the blood of pregnant with premature birth (Tancrède *et al*., 2015), and the reason for the high level of AFP is due to infections occurring in the placenta, causing necrosis of its tissue. Infections of the choroid membranes have a role in the occurrence of premature labor, due to the bacterial infection that is transmitted through the cervix, it penetrates the amniotic sac and reaches the amniotic fluid (Ryan and Black, 2015).

5.1.3. Cystatin C

The results are shown in Table (1) indicated that there was no significant difference in the levels of cystatin C in the amniotic fluid of pregnant with premature birth compared with the healthy pregnant group for all age groups, as cystatin C is a sensitive indicator for assessing kidney function and its level depends on the rate of glomerular filtration. So the level of amniotic fluid does not change in cystatin C for pregnant with premature birth reflects the extent of the development and maturity of the fetus's kidneys compared with healthy pregnant, as there is a change in excretion into the urine with the maturation of the fetus's kidneys and the occurrence of reabsorption. Moreover, the high level of cystatin C in amniotic fluid is associated with an occurrence of pregnancy complications such as pregnancy hypertension and preeclampsia (Sahoo *et al*., 2016).

5.2. Comparison of the activities of selected enzymes in the amniotic fluid of preterm pregnant with the healthy pregnant group

5.2.1: Aminoacylase-1 (ACY 1)

The results shown in Table (2) indicated that there was no significant difference in the activity of the ACY1 in the amniotic fluid of pregnant with premature birth compared with the group of healthy pregnant, that the absence of a significant difference in the activity of the enzyme indicates that there is no abnormality in kidney function or no disturbances in the nervous system of pregnant with premature birth compared with healthy pregnant (Sommer *et al*., 2011). Smith *et al*. (2013) also indicated that both ACY1 and cystatin C are indicators of renal function evaluation (Smith *et al*., 2013).

Table (2)

Enzyme activities in the amniotic fluid of pregnant with preterm birth compared with healthy pregnant.

Measured Enzymes	Enzymes levels in the amniotic fluid according to age groups of pregnant (mean ± SD)					
	First age group (16-25) Year		Second age group (26-35) Year		Third age group (36-45) Year	
	Healthy pregnant (N=24)	Pregnant with preterm birth (N=25)	Healthy pregnant (N=30)	Pregnant with preterm birth (N=22)	Healthy pregnant (N=24)	Pregnant with preterm birth (N=18)
ACY1 (U/L)	5.14± 0.13	4.8±0.11	4.9±0.12	4.9 ±0.14	4.7±0.15	4.7±0.09
LDH (U/L)	98.1±2.6	***232.7±5.4	103.6±3.3	***193±5.8	108± 2.4	***329±4.7
AST (U/L)	9.8 ±0.2	***20.4±1.2	10.3±0.9	**20.9±1.1	10.6±0.7	***28.6±1.5
ALT (U/L)	5.7± 0.1	**16.2±0.3	5.4±0.15	**16.5± 0.3	6.1± 0.1	**17.3±0.3

* Significant difference at P ≤0.05. ** Significant difference at P ≤0.001. *** Significant difference at P≤0.0001.
ACY1: Aminoacylase 1, LDH: Lactate dehydrogenase, AST: Aspartate aminotransferase, ALT: Alanine aminotransferase

5.2.2. Lactate dehydrogenase (LDH)

The results referred to in Table (2) showed a significant increase in the activity of LDH in the amniotic fluid of pregnant with premature birth compared with the healthy pregnant group at P≤0.0001 for all age groups, and these results are consistent with the results of a study a precedent indicated an increase in the activity of the LDH in the blood of pregnant with premature birth (Gurugunti and Sarah, 2016), and another study showed that there was an increase in the activity of LDH in the amniotic fluid of pregnant with premature birth, as well as a sharp increase in the activity of the LDH for pregnant with very premature birth, less than 34 (Umasatyasri *et al*., 2015), and in another study it was observed that there was a decrease in the 37 weeks of pregnancy when the activity of the LDH increased (Shah and Baxi, 2016).

The reason for the high activity of LDH may be due to the bacterial infection that is common in pregnant and that moves from the vagina to the cervix and then reaches the amniotic sac, causing inflammation of the fetal membranes, which occurs in 40% of pregnant with premature birth, and the bacterial infection may be transmitted through the fallopian tubes to reach the uterus (Myntti *et al*., 2016), and in a recent study, Singh *et al*. (2018) indicated that the high level of LDH is associated with the occurrence of complications of pregnancy and the exposure of the fetus to the occurrence of cerebral hemorrhage, cerebral palsy, and lung diseases, so LDH is considered A good indicator for knowing and assessing the incidence of pregnancy complications.

5.2.3. The activities of Aspartate aminotransferase (ALT) and Alanine aminotransferase (AST)

The results are shown in Table (2) indicated that there is a significant increase in AST activity in the amniotic fluid of pregnant with premature birth compared with the healthy pregnant group at a probability P≤0.0001 for the first and third age groups and at a P≤0.001 probability level for the second age group. It was also observed there is a significant increase in the activity of the ALT enzyme for pregnant with premature birth compared with a healthy pregnancy and for all age groups at a probability level P≤0.001, and these results are consistent with the results of previous studies indicating an increase in the activity of the enzymes AST and ALT in the blood of pregnant with premature birth (Zhuang *et al*. ., 2017), and the reason for the high activity of the two enzymes (AST and ALT) is due to the occurrence of necrosis in various tissues of the body, especially the liver, causing impairment in its functions; Because of the increased production of inflammatory cytokines as a result of liver infection (Cui *et al*., 2016), the risk of premature labor increases with the increase in the production of inflammatory cytokines, as Luo *et al*. (2017) indicated that carriers of the hepatitis B virus are more at risk of premature labor. Ekiz *et al*. (2016) also indicated that the incidence of jaundice during pregnancy for various reasons increases the level of hormones to the highest level, especially the hormone progesterone, which stimulates the secretion of substances that contract the muscles of the uterus, as the high level of bilirubin, which is a toxic substance, causes necrosis of liver cells, and then high activities of liver enzymes from AST and ALT beside of the high levels of lipids (cholesterol and triglycerides) are associated with impaired liver function, and low clotting substances put a pregnant woman at risk of bleeding and may lead to fetal death (Zhuang *et al*., 2017)

6. Conclusions

It is concluded from the study that high levels of CRP, cystin C and AFP proteins, some of which can be an indicator of diseases that accompany pregnant women, especially pregnant women with preterm birth. There are clear negative effects about the enzyme variants used to assess liver function in the amniotic fluid of preterm pregnant patients.

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