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REVIEW ARTICLE

PROTEIN KINASE C – ZETA INHIBITORS: A NEW HOPE FOR CONTROLLING MATERNAL INTRAUTERINE INFECTION INDUCED PREMATURE BIRTH.

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Abstract

Premature birth is one of the leading causes of newborn death, estimated 1 in 10 newborn babies. The maternal intrauterine tract infections account for 30-40% of total premature birth. The maternal intrauterine infections are treated with antibiotics, but the neonatal mortality rate has not been decreased. The tocolytic drugs only delay premature delivery for at least 48 hours, but also cause side effects to both mother and the newborn. The protein kinase C (PKC) isoform known as PKC zeta (PKCZ) plays an important role in premature birth. The reports suggest that PKCZ inhibitors have a great potential for being developed as a new drug target for preventing premature birth.

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Introduction:-

Premature birth is one of the leading causes of newborn deaths after pneumonia under the age of five [1]. In USA, the preterm birth rates account for 12-13 per cent. While in European and other developing countries, the preterm birth rates reported 5-9 per cent of total newborn [2]. India contributes to a high percentage of 23.6 % of premature birth related deaths worldwide, which is the highest across 184 countries where this survey was carried out [3]. Therefore it provides a global concern worldwide. It has been estimated that 15 million babies are delivered early every year, before they are mature enough to for survival. The preterm born infant is more susceptible to become

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functionally immature and that results in disability and death [4]. The global premature birth rate is 1 in 10 newborns. WHO has defined premature birth as the delivery of a newborn before completing full 37 weeks of gestation. Premature birth is further sub-divided into following categories:

- Extremely premature (less than 28 weeks of pregnancy)
- Very premature (28 to 32 weeks of pregnancy)
- Moderate to late premature (32 to 37 weeks of pregnancy)

This premature birth related death is highly alarming and needs attention to ameliorate medical conditions in India [5]. Premature birth also causes an economic burden to the society, because of hospitalization of extreme premature infants for intensive care [6]. It has been estimated that one million newborn die due to premature birth every year, while the survivors face a risk for lifetime complications related to gastro-intestinal, neuro-developmental and respiratory system [7]. The probability of having a premature birth is increased to several folds by several risk factors: bacterial infection of cervix and vagina [8], second trimester abortion of pregnant women [9], pregnancy without any interval [10], in-vitro fertilization [11], chronic hypertension [12], diabetes before pregnancy [13], illiteracy and low economic and smoking [14]. Genitourinary tract infection is one of the leading causes of premature birth, accounting for 30-40% of such cases [15]. Our review illustrates intrauterine infections inducing premature birth, available treatment options and therapeutic role of protein kinase C zeta to counter the worldwide problem of premature birth.

Genitourinary infections induce premature birth: clinical and experimental evidences:-

Many researchers believe that premature birth results from extemporaneous activation of common parturition pathway. The common parturition pathway involves myometrium contractibility, decidua activation and/or cervical ripening [16]. The complex physiology amounted with parturition has not yet helped us to the path for solving the intricacies of premature birth. Due to major involvement of many pathological processes, premature birth should be considered as a syndrome and not only “labor before its time” [17]. Microbial infection is one of the important causes of premature birth. Not only one, but more than one pathogenic pathway have been identified which can initiate premature-birth [18].

Watts and colleagues found the relation between bacterial infections with gestational period. They collected amniotic fluid (AF) with intact membranes from idiopathic premature delivery cases. Their results suggested that positive bacterial culture was commonly observed in delivery of women with less than 30 weeks of gestation. While the women with delivery period more than 30 weeks had lesser positive bacterial cultures. It showed an inversely proportional relation between gestation period and positive bacterial culture. The average gestational period of women with premature birth with positive bacterial cultures was 27.5 weeks. The data showed that pregnant women with lesser gestational period (<26 weeks) were more susceptible to bacterial infection. The study also showed that anaerobic and facultative bacterial infections were most commonly found in pregnant women with gestation period at less than 30 weeks. The intrauterine bacterial infection led to broncho- pulmonary dysplasia, respiratory distress syndrome in the neonates and even death in some cases [19].

The most common bacterial species known to be associated with premature birth are *Bacteroides ureolyticus*, *Fusobacterium*, *Mycoplasma* and *Ureaplasma urealyticum*. Dombroski RA and colleagues elucidated the role of bacterial infection in rabbit's pregnancy loss. They inoculated hysteroscopically the pregnant uterine horn of rabbits on day 21(70% of gestation age) with saline solution of *Bacteroides bivius* or *Escherichia coli* or *Fusobacterium necrophorum*. Data showed that inoculation with *E.coli* (92%) and *F. necrophorum* (100%) resulted in higher pregnancy loss; while *B. bivius* (25%) inoculated rabbits resulted in lower pregnancy loss [20].

Manalee Vishnu Surve and colleagues found that Group B Streptococcus (GBS) produces membrane vesicles, which comprises virulence factors such as proteases and pore forming toxins. They challenged chorio-decidual membranes of mice with membrane vesicles ex vivo, which showed collagen degradation. It leads to mechanical weakening and loss of stiffness. Chorio-amnionitis features were mimicked, when GBS membrane vesicles injected into intra-amniotic site. It resulted in increase of pro-inflammatory cytokines and inflammation. It also caused the apoptosis in chorio-decidual tissue. Thus, GBS membrane vesicles can cause chorio-amnionitis, which increases the chances of premature birth [21]. Zahl and Bjerknes injected intra peritoneal the endotoxins of gram negative bacteria into 12-18 days pregnant mice, which resulted in abortion of the embryo [22]. The rate of premature birth is reduced in the

second trimester, if treated against asymptomatic bacterial vaginosis. While no effect is seen on spontaneous premature birth [23]. Therefore, more therapeutic targets are needed to prevent premature deliveries.

Intrauterine infection induced mechanisms and premature birth:-

The previous documented research has revealed that the pathogenic mechanism that induces premature birth involves the activation of hypothalamus–pituitary– adrenal axes (fetus and mother), Inflammation (amnion, chorion and deciduas), decidual hemorrhage, and pathogenic distention of uterine myometrium in mother. The premature parturition can result from the activation of one or more than one of these pathways within months before any clinical manifestation appears for premature birth. However, all pathogenic mechanism follows a common parturition pathway which induces uterine smooth muscle contraction [24].

The intracellular signaling is initiated by uterotonic hormone such as oxytocin, which induces the receptor mediated hydrolysis of a membrane phospholipid, phosphatidylinositol 4,5- bis-phosphate (PtdIns 4,5-P₂) by a phosphoinositide- specific phospholipase C (PI-PLC). This hydrolysis results in the formation of two second messengers, inositol sn-1,2-diacylglycerol (DAG) and 1,4,5-trisphosphate (Ins 1,4,5-P₃). DAG is the physiological activator of Protein kinase C. whilst Ins 1,4,5-P₃ increases the cytosolic calcium concentration by releasing calcium from intracellular stores and activates calmodulin. Phospholipases such as phospholipase A₂ (PLA₂) activity is increased by elevated intracellular free calcium concentration. It results in the synthesis of prostaglandins by providing a source of arachidonic acid. Prostaglandin synthesis and intracellular calcium are determining factors in myometrial contraction. The activation of PKC by DAG results in the phosphorylation of myosin light chain proteins involved in myometrial contraction [25-27]. Thus, PKC plays an important role in signaling pathway of smooth muscle contraction.

Prostaglandin F_{2α} (PGF_{2α}) plays an important role in premature birth. It is synthesized by both membranes of maternal and fetal sides, which binds to prostaglandin F_{2α} receptor (FP). The FP is coupled to PKC activation. PKC activation induces cyclooxygenase-2 (COX-2), which in return induces the synthesis of prostaglandins. This process of prostaglandin synthesis is crucial in fetal membranes prior to premature delivery [28-30]. Recently, it has been found that PKC mediates calcium level independent smooth muscle contraction. PKC phosphorylates CPI-17, an inhibitory protein for smooth muscle contraction. Therefore, it increases the smooth muscle contraction by inhibiting the activity of myosin light chain protein (MLCP) [31-32].

Intrauterine infections trigger inflammatory responses:-

It has been demonstrated that the environmental exposure such as bacterial vaginosis is able to genetically modify the genes that induce premature birth. Heather A. Frey et al. used a single nucleotide polymorphism (SNP) chip to study the effect of environmental factors on premature birth. This pathogenic exposure makes the possible changes in genes which regulate the inflammatory response and make them susceptible for premature birth [33-34]. Intrauterine inflammation response is elicited by recognizing the pattern recognition receptors present on microorganism and induces a pro-inflammatory response. During gestation in humans and animals, researchers have shown the role of intrauterine infection in preterm premature rupture of membrane (PPROM) and premature birth (Figure-1). This results due to pro-inflammatory cytokines like prostaglandins and proteases like leukocyte elastase and matrix metallo-proteinases (MMP). The prostaglandins initiate the uterine contraction and matrix metallo-proteinases (MMP 2 and MMP 9) rupture the chorio-amniotic membranes by degrading structural collagen [35-38].

This cascade involves the release of inflammatory cytokines such as IL-1, IL-1, IL-6, IL-8, tumor necrosis factor alpha and granulocyte colony-stimulating factor (GCSF). Cytokines initiate the prostaglandin synthesis. Neutrophils promote chemotaxis and result in release of matrix metalloproteinases and other bioactive molecules [39-43]. During pregnancy, vaginal/cervical fibronectin is responsible for the detachment of placenta to uterus. It comes under the family of trophoblast proteins. Inflammation induces proteolysis and increases the chances of placental-uterine detachment. This results in the release of vaginal secretions increasing the chances of premature birth by 40-60 folds. Thus, vaginal/ cervical fibronectin is indeed an important factor in the prediction of premature delivery [44]. The first cytokine associated with pathogen induced premature birth is IL-1 produced by women deciduas. IL-1 level is found to be more in pregnant women with premature birth and stimulates the production of prostaglandins by women amnion and deciduas. It is able to stimulate myometrial contraction. While the researchers have shown that IL-1 type I receptor knockout mice also show premature birth after exposure to infection. Chorioamnionitis involves the dominance of interleukin-6 in decidual cells [45-46]. Often intrauterine infection is asymptomatic and

occurs at an early stage of gestation [47]. Thus, the markers associated with intrauterine infection in women would serve as helpful tool to prevent the premature delivery.

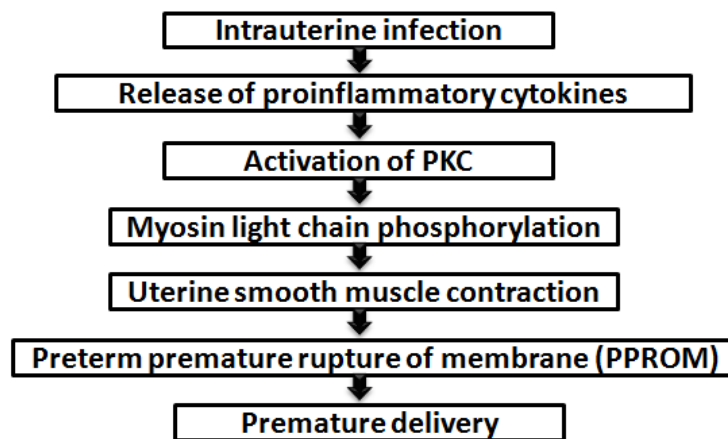


Figure 1:- The mechanism of parturition, showing the role of proinflammatory cytokines and PKC

Limitations of tocolytic agents in treatment of premature delivery:-

The maternal intrauterine bacterial infection has been treated with antibiotics like clindamycin or metronidazole for prophylaxis. But the mortality rate or premature birth rates have not been decreased [48]. The tocolytic agents are known to inhibit myometrial contraction and used for treating premature delivery. The studies show that these tocolytic agents only delay premature delivery for at least 48 hours and allow mother to transfer for intensive care unit of neonates. These tocolytic agents do not show any improvement on the side effects of premature birth on infants [49]. The two main pathways that are blocked while preventing premature delivery include the tocolytic agents which blocks myometrial stimulant and alteration of intracellular messengers. The tocolytic drugs which inhibits the action of myometrial stimulant includes oxytocin antagonists (Atosiban) and prostaglandin synthesis (Indomethacin) inhibitors. While the drugs affect the action of intracellular messengers include β -Adrenergic-receptor agonist (ritodrine hydrochloride) and calcium channel inhibitors [50-52]. The Food and Drug Administration (FDA) has approved only ritodrine as tocolytic agents. The pharmacological information is limited about other tocolytic agents. Therefore researchers are comparing alternative options of drugs with this gold standard known as ritodrine[53].

The corticosteroids given to mother before delivery decreases the side effects of preterm birth by 40 per cent. This approach decreases the risk of intraventricular hemorrhage and respiratory distress syndrome in preterm born infant. The combined tocolysis and steroids approach allows accelerated infant maturation [54-55]. In a clinical study, Atosiban (oxytocin receptor antagonist) caused lesser adverse effect than ritodrine hydrochloride (β adrenoceptor agonist). While with placebo, Atosiban showed no decrease in complications of premature birth such as respiratory distress syndrome [56,57]. While Roel de hues and colleagues examined the use of these tocolytic agents with serious maternal complications [68]. 1920 women from 28 different hospitals in Belgium and Netherland were treated for premature delivery with different tocolytics. Out of 1920 women, 1327 undergone single course of tocolytics agents (69.1%), 282 received sequential tocolytic course (14.7%) and 311 underwent for combined tocolytic course (16.2%). Out of 1920, 0.7% showed adverse effects. Compared with relative risk of atosiban, the relative risk with β adrenoceptor agonist single treatment was 22.0(95% confidence interval 3.6 to 138.0) and calcium antagonist single treatment was 12 (1.9 to 69). Multiple tocolytic treatment led to five serious adverse effects (1.6%). They found that atosiban and Indometacin were the only tocolytic agents that were not associated with adverse effects. While the use of β adrenoceptor agonist and multiple tocolytic treatment was associated with adverse effects (Figure-2).

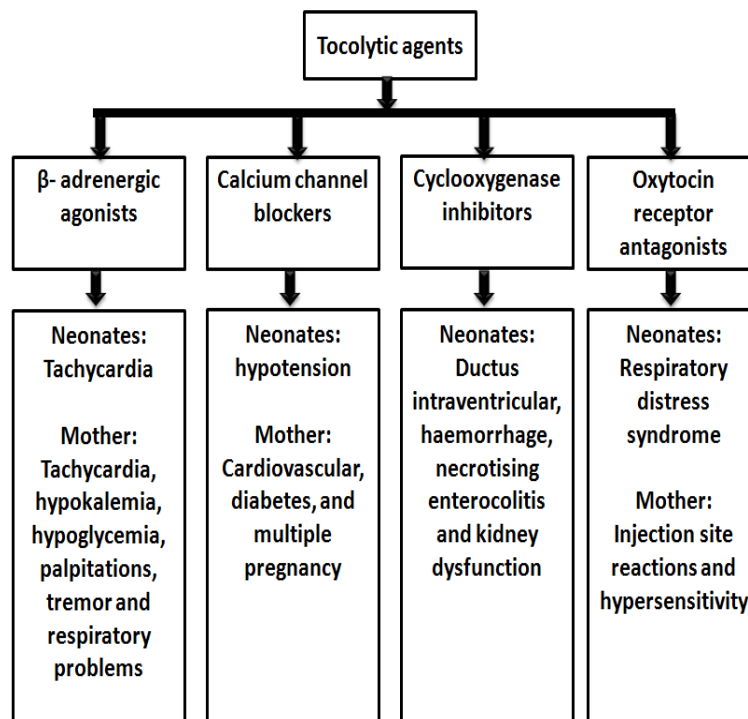


Figure 2:- Different types of tocolytic agents and their side effects

The use of these tocolytic agents is controversial, due to its side effects. Till date no satisfactory study has been carried out that will compare the efficacy of these different tocolytic agents and their adverse effects on routine clinical pregnancy. If its efficacy is checked on small populations, it may have adverse effect on different geographical regions. The tocolytic treatment may be more helpful if used early in the labour. Therefore it is important to interpret tocolytic therapy clinical trials. The search for a new biomarker for premature birth had no significant impact in decreasing the premature birth. Therefore, the approach comprising novel treatment option with biomarker activity may help us in treating premature birth morbidity and related mortality.

Prediction of premature birth:-

Our main focus is to find out the new reliable and promising targets in common pathways which would help in preventing premature delivery and reducing the morbidity and mortality rate. The clinical molecular markers are not precise enough to predict the risk of premature birth. While fetal fibronectin, biochemical markers (cervical dilation, shortening, softening, uterine contraction) are used alone or together to predict the chances of premature birth. But the uterine contraction is used as one of the major indicators of initiating the process of parturition leading to premature delivery [59]. Thus, inhibiting uterine contraction is one of the main targets for successfully preventing premature birth.

Gomez and colleagues research have revealed that bacterial infection induced newborn delivery is in control of host [60]. They argued that if intrauterine environment is under the bacterial infection and survival of the fetus is in danger, the host system minimizes this risk by choosing the option of premature birth. Thus, premature birth can be considered as the similar phenomenon like the activation of macrophage system against any pathogen. While Wanjuan Tan et al. revealed that bisphenol A exposure induces premature birth [61]. Bisphenol A is used to manufacture polycarbonate plastics. It has been isolated from pregnant women sera, breast milk and placental tissue. They found that PKC ζ/λ and δ activated immune responses in placenta of mice treated with bisphenol A. They also observed an increase in the level of estradiol, corticotrophin releasing hormone, testosterone, placental PKC ζ/λ and δ in mice as bisphenol concentration rises. Yanfei wang et al. also demonstrated that fungal toxin known as aflatoxin B1 also induces premature birth [62]. The pregnant mice were treated with 0.5mg/kg aflatoxin B1. They observed increase in the level of CRH, estrogen and progesterone while increasing the higher doses of toxin. They have also suggested that PKC activation is largely responsible for this premature birth and involved in signal transduction process.

PKC Zeta in premature delivery:-

The aim of our study is to investigate the importance of PKC in uterine contractility. Recently, PKC inhibition has been shown to prevent infection induced premature birth. PKC main function is to phosphorylate the myosin light chain proteins, which results in myometrial contraction [63]. PKC inhibitors provide a new hope for controlling infection induced premature birth. Protein Kinase C (PKC) belongs to serine/threonine kinase family and is shown to be involved in a wide range of cellular processes such as adhesion, migration, polarity, anoikis, division, proliferation and survival. PKC family involves 12 serine/ threonine kinase. On the basis of their activation mechanism, these are divided into three subfamilies. The diacylglycerol (DAG) and calcium activates conventional PKCs (α , $\beta 1$, $\beta 2$, γ), DAG activates novel PKCs (ϵ , δ , θ , η) but not activated by calcium, and both calcium or DAG cannot activate atypical PKCs (ζ and λ) [64]. The PKC plays an important role in signal transduction which facilitates the cross talk between different signaling pathways.

John J. Morrison and colleagues tried to elucidate the mechanism operating in oxytocin-induced uterine contraction during delivery [65]. The study demonstrated that oxytocin stimulated contraction was reduced by both PKC inhibitors such as RO 31-8220 and staurosporine, which are structurally distinct. At higher concentration (10~5 M), an approximately same amount of oxytocin stimulated contractility was reduced by both PKC inhibitors. At lower concentration (10~7 and 10~6 M), RO 31-8220 was found to be more involved in oxytocin stimulated contractility than staurosporine. They found that tyrosine kinase inhibitor: genistein also reduced oxytocin stimulated contraction. Compared to PKC inhibitors, higher concentration of genistein was required to inhibit oxytocin induced myometrial contractility. These results suggested that PKC plays an important role in signal transduction pathways of cytokines, growth factors and hormones, which are involved in the modulation of oxytocin induced contractions during pregnancy. Researchers have shown the importance of endothelin-1 in premature birth. Endothelin-1 plays an important role in smooth muscle contraction and acts as a pro-inflammatory mediator. Firstly, phospholipase C is activated by endothelin-1; in turn it activates PKC and sphingosine kinase (SphK). SphK leads to the activation of Rho kinase, which results in myometrial contraction [66-70].

Qing Chun Shao and colleagues used the consensus scoring strategy to find out binding strength of inhibitors into PKC zeta active pocket. The 8 out of 32 top score inhibitors were tested for their promising candidature against PKC zeta. However, Five inhibitors i.e. PIM1 inhibitor myricetin ($IC_{50} = 1.7 \pm 0.4 \mu M$), CDK6 inhibitor fisetin ($IC_{50} = 58 \pm 9 \mu M$), CDK9 inhibitor flavopiridol ($IC_{50} = 108 \pm 17 \mu M$), promiscuous kinase inhibitor staurosporine ($IC_{50} = 0.019 \pm 0.004 \mu M$) and PknB inhibitor mitoxantrone ($IC_{50} = 280 \pm 47 \mu M$) showed higher or moderate inhibitory activity on PKC zeta. Other three inhibitors, dasatinib, sunitinib and rho inhibitor fasudil did not show any observable activity [71]. These PKC zeta inhibitors may be helpful in inhibiting the premature delivery. While the Patel and Doerksen developed a database of protein kinases with its inhibitors [84]. These inhibitors were grafted into the active pocket of PKC zeta by using structure superimposition approach and the inhibitors with higher affinity were evaluated by using consensus scoring technique. Kinase assay has been also used to measure the inhibition potential of several candidates against PKC zeta by theoretical analysis [72].

Isabelle Eude-Le Praco et al. studied the role of different PKC isoform in promoting myometrial contraction [73]. They observed that antisense- oligonucleotide against PKC ζ and inhibitory peptide specific for PKC ζ resulted in inhibiting the ET-1 induced myometrial contraction. While the Inhibitors against other PKC isoforms did not show any effect on ET-1 induced myometrial contraction. The PKC ζ may be affecting the binding properties or phosphorylation of actin that leads to premature delivery.

Conclusion:-

Genitourinary infection is associated with more than 30-40% of premature birth cases and reports suggest that PKC zeta involvement in promoting women uterine contraction are inevitable. Intrauterine infection leads to the release of inflammatory cytokines which in turn modulates the oxytocin induced contraction during pregnancy. It has been suggested that pathogen induced premature birth need not only antibiotic treatment but also IL-10, anti-oxidants macrophage inhibitory factor and TNF alpha blockers. It is also found that IL-1 and TNF alpha blockage can decrease the chances of premature birth. But till not no significant improvement has been done in preventing premature delivery. PKC zeta inhibitors have great potential for being developed as a new target for preventing premature delivery. It would be helpful in reducing the side effects of tocolytic agents. While the other vital mechanisms yet to be explored.

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