Cortisol, CRH and 11βHSD interrelation mechanisms in the presence of stress: effects on fertilization and fetal development, and review of molecular assessment methods

Summary

Stress causes cortisol levels in the blood to rise, altering the expression and activity of its regulatory agents – corticotropin-releasing hormone (CRH) and 11β-hydroxysteroid dehydrogenase (11βHSD). In this paper, the effects of cortisol, CRH, and 11βHSD on conception and fetal development are analyzed, including review of relevant research, assessment methods, and potential future studies. Within a woman’s body the ovaries and the placenta independently control cortisol, CRH, and 11βHSD concentration. However, this hormonal balance can be disrupted by the high cortisol concentration in the blood, consequently decreasing chances of conception and altering fetal development. High pre-conception intraovarian cortisol levels, a low cortisol/cortisone ratio, and oxidative 11βHSD activity have been found to negatively affect fertilization, especially concerning in-vitro procedures. During gestation, high levels of maternal cortisol can increase the amount of placental CRH, potentially effecting substance transport across the womb, altering fetal gene expression, and increasing the risk of abnormalities in mental development, cardiovascular diseases in offspring, or premature birth. These negative stress-related outcomes emphasize the importance of cortisol, CRH, and 11βHSD screening. Several suitable molecular techniques are reviewed in this paper. These include immunoassays, liquid chromatography with tandem mass spectrometry, and the polymerase chain reaction, applied to saliva, urine, blood plasma, hair, follicular fluid, or amniotic fluid samples. Such screening may play an important role in successful IVF procedures as well as avoiding fetal physiological impairments. However, further investigation is needed regarding the relationship between maternal stress and the concentration of cortisol, CRH and 11βHSD in the ovaries and placenta.

Keywords: maternal stress, cortisol, 11beta-hydroxysteroid dehydrogenase, fetal development, fertilization, laboratory assessment techniques.
INTRODUCTION

Scientists agree that maternal stress during gestation negatively affects the development of the fetus [1, 2], triggering the need to further examine this topic. Most of the studies thus far have investigated the effects of the major stress hormone, cortisol, and regulating agents such as corticotropin releasing hormone (CRH) and 11β-hydroxysteroid dehydrogenase (11βHSD). The manner in which these substances act in the presence of stress—and how they affect the physiological reactions, ovarian follicular fluid of women, and the placental environment—have been clearly described in a number of research studies [3–5]. It was found that the altered secretion of stress hormones and proteins affects the process of fertilization and gestation, and can result in physiological and neurological impairments of the offspring [6]. In the majority of studies, specific methods to evaluate levels of cortisol and its regulators were used. These included immunossays, liquid chromatography with tandem mass spectrometry (LC-MS/MS), and the polymerase chain reaction (PCR). Researchers can assess urine, saliva, blood plasma, and hair samples from pregnant women. While monitoring the effects of stress the possibility of conceiving and on prenatal programming, researchers use ovarian follicular fluid and amniotic fluid, respectively. This paper addresses the effects of stress on conception and fetal development, from basic mechanisms to assessment techniques, providing examples of important research findings and identifying areas for further investigation.

CORTISOL PRODUCTION IN THE HUMAN BODY AND TRANSPORT ACROSS THE PLACENTA IN THE PRESENCE OF STRESS

Cortisol (hydrocortisone) is a glucocorticoid hormone regularly secreted by the adrenal gland. The release of cortisol is triggered by the adrenocorticotropic hormone, excreted in the anterior pituitary gland, which is stimulated by CRH expressed in the hypothalamus. Despite being one of the most important glucocorticoids in controlling energy and metabolism, after entering the blood stream CRH serves the body by reducing stress caused reactions. In the presence of emotional or physical stressors, cortisol levels increase the amount of blood glucose, fatty acids, and amino acids, and raise blood pressure. These effects result from changes in the gene activity in the target cells. However, if the stress is severe, or lasts for an extended amount of time, high levels of cortisol tend to depress the immune system and inflammatory responses. Additionally, excessive secretion of cortisol is known to lead to deficiencies in cartilage and bone formation, and impaired cardiovascular, neural, or gastrointestinal function [4].

During the course of pregnancy, genes coding CRH and other stress hormones are expressed not only in a woman’s hypothalamus, but also in her placenta. Both human CRH genes, placental CRH (pCRH) and hypothalamic CRH (hCRH), are located on chromosome 8 at 8p13 location. pCRH is controlled by different gene expression and regulation agents than hCRH [7], and serves the function of triggering the excretion of cortisol from the mother’s adrenal gland. Additionally, while cortisol stimulates a negative feedback mechanism in the hypothalamus (decreasing the expression of CRH), this stress hormone acts as an activating agent for the expression of pCRH (positive feedback) after easily passing into the placenta [1]. As a result of this positive feedback loop, the more pCRH is released, the more cortisol is excreted. Thus, the concentration of maternal cortisol (together with pCRH) increases during gestation [6]. Despite the fact that pCRH is secreted in the maternal environment [8], the increased levels do not make the woman feel more alert. Due to this regulation, the maternal HPA axis becomes less responsive to stress as pregnancy progresses [9]. Even though the mother may not consciously experience the effects of severe stress, it must be taken into consideration that substance transport across the placenta occurs both ways. Research shows that excessive amounts of maternal cortisol can cross the placenta and contribute to an average 33.5% variance in fetal cortisol levels [10]. However, placental cortisol remains several times lower than maternal because of oxidative 11βHSD enzyme activity.

IMPORTANCE OF 11βHSD FOR CORTISOL LEVEL REGULATION

Regulation of cortisol is known to rely on 11βHSD [3, 11]. There are two isoforms of this enzyme: type 1, coded by the HSD11B1 gene (located at 1q32-q41), and type 2, coded by the HSD11B2 gene (located at 16q22) as described by NCBI. 11βHSD1 requires NAD(P)H and generates cortisol, thereby increasing metabolism. However, it can also convert cortisol into its inactive form, cortisone, by combining with NAD(+) 11βHSD2 requires NAD(+) and has been found to oxidize cortisol. Both 11βHSD1 and 11βHSD2 enzymes take part in controlling the amount of cortisol available to bind corticosteroid receptors. Evidence of such regulation has been detected in several tissues (e.g., kidneys, liver, etc.) as well as in women’s ovaries and placentas [12, 13]. Intraovarian and placental 11βHSD type 1 and type 2 enzymes mainly perform an oxidative function, and have an important effect on fertilization and pregnancy. The regulation of intraovarian cortisol levels rely on granulosa-lutein cells located in the ovarian follicles. Granulosa cells express 11βHSD2 mRNA during the follicular phase, and switch to 11βHSD1 mRNA during luteinization while undergoing the ovarian cycle [5]. Regulation of the cortisol levels affecting the fetus relies on 11βHSD expression in the placenta. Oxidizing enzymes are the most active in the early gestation period, and activity decreases as pregnancy proceeds. This mechanism plays an important role in the formation and maturation of the fetal lungs, central nervous system, as well as other organs and tissues at the time of delivery [6].

THE EFFECT OF STRESS ON FERTILIZATION

Examining the outcomes of assisted reproduction procedures, scientists have documented the importance of intraovarian cortisol. When investigating in vitro procedures, it was observed that cortisol metabolism is related to the establishment of pregnancies [13]. Studies showed that significantly lower cortisol and cortisone levels were detected in ovarian follicular fluid (FF) of women who conceived through IVF-ET procedures, compared to those who did not. The ratio of cortisol/cortisone was higher during the successful conception cycles and lower in the unsuccessful procedures (the reported breaking point ranged from 7.7 to 11.4) [11, 14]. In cases where patients were treated with human chorionic gonadotropin (hCG) be-
fore the in vitro fertilization (IVF), granulosa-lutein cells failed to oxidize cortisol into cortisone through released 11βHSD, thereby increasing the possibility of establishing clinical pregnancy. However, no research was found that specifically investigated the variability of intraovarian cortisol in relation to maternal stress and anxiety. If maternal cortisol is able to reach the follicular fluid and increase the amount of ovarian cortisol – thereby changing the cortisol/cortisone ratio and affecting ovarian 11βHSD activity – then reduction of maternal stress would be an important factor in infertility treatment.

Research also shows that stress and increased maternal cortisol during the late stage of follicular development impairs the growth of oocytes and the in vitro formation of blastocysts, and negatively affects the weight of the newborn [15]. These results were observed in both hCG injected and non-injected subjects. Furthermore, as reported by E. J. Mulder et al. (2002), anovulation, oligomenorrhea, and low conception rates were identified among chronically stressed patients [2]. Such findings increase the likelihood that stress induced maternal cortisol increases may disrupt the concentration and ratio of cortisol and cortisone, as well as 11βHSD activity inside the ovaries, thus impairing ovarian function.

It is clear that non-oxidative 11βHSD activity in the ovaries during the luteinization phase, and low maternal cortisol levels, have a positive effect for conception. Addressing this problem could have favorable implications for families suffering from infertility issues, especially because the nature of infertility itself tends to raise anxiety and stress.

THE EFFECT OF STRESS ON MATERNAL CORTISOL AND THE FETUS

The amount of cortisol naturally increases during gestation. However, emotional imbalance may disturb the natural process. Higher amounts of cortisol during the period of stress exponentially increase the concentration of pCRH [16], which means it turns the pCRH positive feedback loop in the placenta earlier than it normally would. Research implies that the growth and development of the fetus is regulated by pCRH, as it alters the transport of various substances across the placenta [17]. Consequently, rapid exponential growth of pCRH, induced by stress, may alter the development of an embryo.

During fetal development, the gestational timeline is very important: organs and tissues develop in a specific sequence. As a result, the timing of a pregnant woman’s exposure to stress determines which process of development may become affected [18]. For example, it has been reported that stress during the first trimester of pregnancy is associated with lower birth weights compared to those infants whose mothers experienced stress after the first three months of gestation [2]. This fact is highly consistent with the timing of fetal development, as major morphological impairments are formed during the first trimester [19]. Moreover, various fetal alterations in response to stress increase the risk of cardiovascular, metabolic, or muscular disorders that may ultimately reduce the life span of child [20, 21]. A recent study by D. Nesan and M. M. Vijayan (2012) on fish embryos revealed that morphological changes in the presence of stress are determined by altered gene expression. Elevated basal cortisol levels during embryogenesis were found to increase the possibility of heart deformities in zebrafish. This correlated with suppression of cardiac genes such as nkx2.5 (cardiac transcription factor), cmcl1 (cardiac myosin light chain 1), tntn2a (cardiac troponin T2a), and atp2a2a (muscle calcium transporter ATPase). This effect of artificially increased cortisol was reported to mimic the outcomes of maternal stress on the early embryo: decreased cardiac performance and a reduced chance of survival for the offspring. Despite the fact that this study was performed with fish embryos, the mechanistic link between maternal stress and physiological impairments of offspring has significant implications for studies in humans [20]. Research designed to learn more about gene expression in developing mammalian embryos may reveal new insights applicable to maternal stress studies.

Additionally, research shows that fetuses may have the capacity to adjust to stressful environments by altering regular development of the nervous system, especially the volume of gray brain matter [1]. This can also result in cortisol induced spontaneous abortions or premature births. Accordingly, the premature expression of pCRH, by high stress and anxiety in a pregnant woman, can cause impairments in physiological, motor, or cognitive functions of a newborn [2, 22, 23]. Due to these reported impairments during fetal development, screening pregnant women for elevated cortisol levels and activity of placental 11βHSD, especially during the first trimester, may be important in improving pregnancy outcomes.

SCREENING METHODS
Cortisol assessment techniques for pregnant women

Before measuring stress outcomes for intraovarian or placental environment, it is important to confirm that the woman herself is affected by stress. For this reason, cortisol levels are monitored. However, it must be taken into consideration that this hormone peaks in the morning and gradually declines throughout the day [24]. To assess cortisol or cortisone in a fluid a researcher can use immunoassays.

The radioimmunoassay (RIA) method relies on radioactively labeled antigens binding to the specific antibodies. This method is extremely sensitive, specific, inexpensive, and relatively easy to perform [25, 26]. It is still used primarily for learning purposes, and is not common in everyday clinical practice. Two other methods, Enzyme immunoassay (EIA) or Enzyme-linked immunosorbent assay (ELISA), are performed more often. In EIA/ELISA, enzyme coupled receptors are used instead of radioactive substances. These technologies have become specialized for various fields, including reproductive endocrinology, to assess hormone levels in plasma [27]. The immunoassay method has been criticized for being too sensitive, as antibodies cross-react with steroid compounds which are considered to be similar (e.g. 21-deoxycortisol and corticosterone). In order to eliminate this problem, one could use chromatography (e.g. LC-MS/MS). The LC-MS/MS method has its drawbacks as well, since cortisol analysis by this method may reflect interferences of isomers or isobars, indicating a need for efficient chromatographic separation by an experienced technician. However, LC-MS/MS performed by professional staff is preferable for precise cortisol assessment, especially for subjects taking specific medication that might cause cortisol analogues to accumulate [28].
Utilizing a variety of samples to assess cortisol

To check if a pregnant woman is affected by stress, various types of samples can be used, namely saliva, urine, blood serum, and hair. The use of blood serum as a sample material is not the preferred method for stress detection. It measures not only free, but also bound cortisol – which can result from medications.

Cortisol amounts caused by stress can be reliably assessed from a saliva sample. A saliva evaluation method has some advantages over the others, as the samples are easy to collect and store (e.g. keep the samples at room temperature for up to 7 days) [28]. A. F. Bell et al. (2012) used saliva samples together with EIA kits to measure cortisol in women right after giving birth. The enzyme bound cortisol, in this case, was detected by a Microtitre plate reader at the specific absorbance right after the reaction with chromagen. During the study, EIA testing was compared to RIA testing from the same company, resulting in a positive correlation and proving the reliability of both methods [29].

Another easy-to-administer method relies on 24-hour urine samples. Collected throughout the day, they are usually assessed by chromatography techniques: for example, via high-pressure liquid chromatography assay [30] or quantitative gas chromatography – mass spectrometry [31]. 24-hour urine cortisol measurements are considered a good indicator for acute or chronic stress, if performed periodically. Additionally, saliva and urine cortisol tests assess the strength of real-time stress.

A relatively new and promising method, evaluating cortisol from hair samples, serves to obtain a biological marker to report long-term, chronic stress [24]. Hair cortisol assay was designed using modified salivary ELISA, where methanol is used to extract this hormone from the hair sample [32]. It is recommended that cortisol levels be checked while using hair assay, as the increased activity of 11ßHSD2 in the outer hair follicle can lower the amount of detected cortisol [33].

Measuring cortisol in saliva, urine, or hair samples provides results about the severity of stress affecting the mother's body. These measurements might be combined with the information obtained from cortisol and 11ßHSD screening using follicular and amniotic fluid samples, as the overall results can be very relevant to fertilization and the prenatal growth of the fetus.

Evaluating stress outcomes on intraovarian and placental environment

There are two ways of assessing stress outcomes for the intraovarian or placental environment. The first relies on monitoring levels of cortisol (sometimes together with cortisone). The second measures enzymatic activity of the 11ßHSD enzymes. In cases of maternal anxiety or stress, levels of 11ßHSD drop significantly and cortisol is poorly oxidized into cortisone [3, 5]. Cortisol and cortisone concentrations can be evaluated from the intraovarian or amniotic fluid [11, 21] using the same techniques as those used to assess the saliva, urine, blood plasma, and hair samples obtained from women. This includes RIA, EIA/ELISA, and LC-MS/MS. For example, A. E. Michael et al. (1999) measured cortisol and cortisone concentration from the ovarian follicular fluid via RIA and found lower levels in successfully fertilized women. K. Bergman et al. (2010) used the RIA method to evaluate cortisol amounts from amniotic fluid for women during midterm pregnancy while assessing their anxiety levels [21]. A significant relationship between prenatal stress of the mother and cognitive development of the infant was found, indicating an increased risk of poor adjustment to the environment, as well as learning difficulties, for the offspring.

11ßHSD enzyme expression and activity is usually monitored by PCR techniques. Type 2 11ßHSD is assessed more often because of characteristics and oxidative properties. This method relies on collected samples of tissue or fluid (e.g. placental tissue). RNA can be isolated from the sample using TRIzol reagent and then cDNA libraries are created using reverse transcriptase. Quantitative or semi-quantitative RT-PCR is then performed. For the human HSD11B2 gene scientists use two primers: forward 5’-GCTCATACGGGCGTGTA-3’ and reverse 5’-GGGTGTCCAAGAACACT-3’ [3]. To avoid errors it is important to perform the verification procedure using normalization to another selected protein. When gene expression rates are already present, another important factor is their activity measurement. If the expressed protein is inactive, it cannot perform the essential function.

To analyze protein activity from tissue or fluid the sample homogenate is washed of debris and the chromatography procedure is then performed. In cases where the activity of the enzyme is altered, results of both procedures are analyzed for significant relationships that show whether transcriptional or post-transcriptional/translational factors were responsible. After using this method, K. J. O’Donnel et al. (2012) stated that prenatal stress in late pregnancy suppresses the amount of 11ßHSD2 in the placental tissue, resulting in raised cortisol levels affecting the fetus [3]. As mentioned earlier, this may result in premature birth and lower volume of gray matter in the infant’s brain, which can lead to decreased mental abilities.

DISCUSSION AND FURTHER RESEARCH

As discussed in this paper, levels of cortisol elevated by stress have a significant importance on the health of a woman, conception, and fetal development. Although the reviewed studies explain the mechanism of how cortisol is regulated, monitored, and related to fertilization and prenatal programming, there are several areas in need of further investigation.

First, A. E. Michael et al. (1999, 1995) demonstrated that high intraovarian cortisol levels and poor metabolism by 11ßHSD are related to the success rates of IVF conception-cycles [11, 13]. However, no animal or human research was found examining this topic with subjects trying to conceive without medical intervention. In this paper, the question was raised whether some amount of stress-elevated cortisol can be transported into the follicular fluid, and therefore change the 11ßHSD activity. One way to find an answer could be a study assessing the amount of cortisol, cortisol/cortisone ratio, and the expression and activity of 11ßHSD in the ovarian follicular fluid [14] when women report exposure to high levels of stress. Data from such studies could establish a link between stress and the likelihood of conceiving, which may have a positive effect on infertility treatment.

Another area worth studying is the effect of acute stress on the embryo/fetus specifically in the first trimester of gestation. As noted by T. Sadler (2003), E. J. Mulder et al. (2002), M. V. Johnston et al. (1995), the devel-
opining fetus is highly vulnerable during the first trimester [2, 18, 19]. During that period, stress affects the growth of a fetus and can even result in morphological defects of the infant. Many other studies also document physiological and neurological impairments of a newborn in response to prenatal stress [21, 22]. These studies have revealed the overall effect of stress without elaborating on the specific details of which structures of fetuses were developing at the time the subject was affected by stress. Further research could be performed on laboratory animals in controlled experiments monitoring cortisol levels of a pregnant females (to check if the animals monitoring cortisol levels of a research could be performed on laboratory subject was affected by stress. Further research would be especially beneficial if performed with mammalian embryofetuses, as findings may open completely new insights relevant to the human stress studies.

The questions raised in this paper clearly reinforce the importance of molecular techniques in the related research. While all the previous studies examining stress effects on cortisol secretion, fertilization, and prenatal development rely on RIAs, EIAs/ELISAs, chromatography methods or PCR's, it is obvious that future research will need to be based on more complex and detailed molecular analysis. This emphasizes the necessity of synthesizing the available knowledge, results, and methodologies described in the scientific papers, to review stress related biological mechanisms affecting conception, fetal programming and growth, as well as available techniques for investigation.

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**Santrauka**

**KORTIZOLIO, CRH IR 11β-HSD TARPUŠAVIO RYŠIAI STRESO MECHANIZMĖ: POVEIKIS APAVSNIMUI, VAIASIAUS VYSTYMUSI IR TYRIMAMS TAIKYTINĮ MOLEKULINĮS BIOLOGIJOS METODŲ APŽVALGA**

**Ieva Masliukaitė**

Įvertindamas nuotraukos struktūros, 11β-4-ketozidų dehydrogenazės (11βHSD) expresija. Straipsnyje, aptariant įvairių mokslinių tyrimų rezultatus, apibūdinamas kortizolo, CRH ir 11βHSD poveikis moters vaisingumui bei prenatalinai vaisingumai raidai, aprasomi tinkami laboratoriniai tyrimo metodai ir pateikiamos įvairios būtinos tyrimų temos. Mėnesiorganizu kiaušinėse ir placecentos savarankiškai reguliuoja kortizolo, CRH ir 11βHSD koncentracija, Streso meta

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