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PCOS model: Apoptotic changes and role of vitamin D

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ABSTRACT

Aim: This research was aimed to observe apoptotic marker (caspase 3) levels in different groups after treatment with vitamin D in an animal model after inducing polycystic ovary syndrome (PCOS) with dehydroepiandrosterone (DHEA).

Materials and Methods: 30 pre-pubertal female sprague dawley dams were recruited. The animals were distributed 10 each in control, PCOS and vitamin D treated groups. In control group, 0.2 ml of sesame oil was given. PCOS group was administered DHEA by the daily dose of 6 mg/kg for 30 days. In vitamin D treated group, animals were injected 6 mg/ kg/day DHEA daily and 120 ng 1, 25(OH) 2D3/100 g subcutaneously once a week. The occurrence of programmed cell death in PCOS by apoptotic marker (caspase 3) levels.

Results: The results of this study showed significant weight gain, obesity, changes in caspase 3 levels in PCOS group as compared to control and vitamin D treated group.

Conclusion: Administration of vitamin D (120 ng 1, 25(OH) 2D3/100) reduced body weight as well as improved the caspase 3 levels in PCOS induced animals. The results support the effect of vitamin D treatment for metabolic and reproductive characteristic features in PCOS females.

Keywords: animal model, DHEA, polycystic ovary syndrome, vitamin D, caspase 3

INTRODUCTION

Polycystic ovary disorder is a complex endocrine syndrome that mainly affects women of childbearing potential [1]. It is a syndrome of multiple systems, with 6-10% of women of reproductive age in the world [2]. It is characterized by irregular menstruation, overweight, hair loss, infertility, hyperandrogenism, and ovulation [3].

In polycystic ovary syndrome (PCOS), infertility is caused by poor follicle growth and anovulation, resulting in irregular menstrual periods and pregnancy disorders as well as implantation disorders and pregnancy loss [4] .Furthermore, PCOS has an increased risk of obesity and cardiovascular abortion due to metabolic factors. It is estimated that poor oocyte quality and a variety of endometrial morphologies can lead to miscarriage [5].

One of the underlying mechanisms is disrupted endometrial function due to hormonal imbalance which results in altered protein expression and affects normal cell proliferation and apoptosis. One of these proteins is caspase 3, which shows a vital role in cell death process. Caspase 3 is a pro-apoptotic protein which plays a significant role during the process of programmed cell death. Thus, it is necessary for chromatin clumping, breaking down of DNA, loss of cell membrane's integrity in addition to the production of apoptotic bodies [6]. This apoptosis in ovarian granulose cells result in oocytes having poor embryonic growth [7]. Studies have found significant differences in terms of caspase 3 activity between subjects with normal and abnormal endometrium [8]. In addition, some inherited and environmental causes are thought to be elaborate in pathogenesis [9]. Among treatment options vitamin D has been found to recover endometrial function in patients with PCOS [10] and cardiac dysfunction in murine model of PCOS [11].

In recent years, the relationship between infertility and vitamin D deficiency has been exposed. Vitamin D receptors are found in the endometrium, placenta and ovary [12]. Alterations in calcium absorption due to vitamin D deficiency interfere with follicular growth, menstrual regularity and fertility. Numerous studies show that serum vitamin D concentrations are <20 ng/mL in 65-86% of people with PCOS and that treatment with vitamin D supplementation may have valuable properties on insulin resistance and menstrual disturbances [13, 14]. Conversely, it is not widely expressed for the exact mechanism vitamin D makes these properties [15] and excessive vitamin D leads to intoxication [16]. Various treatment methods are applied to patients with PCOS to relieve these signs.

This research was aimed to observe apoptotic marker (caspase 3) levels in different groups after treatment with Vitamin D in an animal model after inducing PCOS with dehydroepiandrosterone (DHEA).

Table 1. Data of the average weight, number of estrous cycles, and caspase 3 activity levels in three different group of (n=8) rats

	Normal control	PCOS control	Test group
Average weight of rats	83.83	103.34	84.90
Average number of estrous cycle in rats (proestrus, estrus, metestrus, & diestrus)	4 10 7 5	5 2 9 4	5 4 7 4
Average levels of Caspase 3 activity in rats	0.7975	0.98375	0.78125

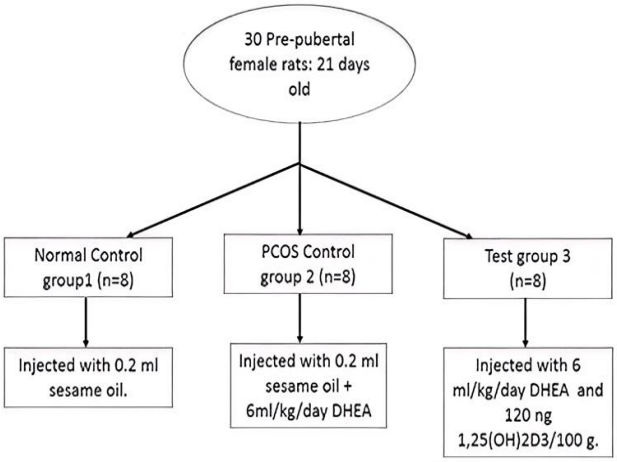

Figure 1. Grouping and PCOS Induction of the study

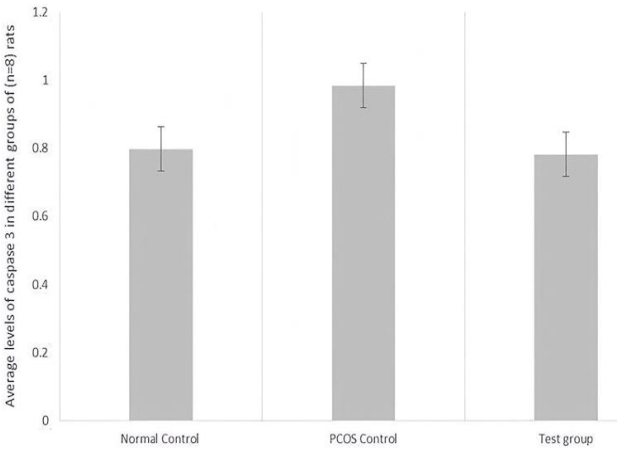

Figure 2. Increased body weight recorded in three different group of rats

MATERIALS AND METHODS

Table 1 shows the data of the average weight, number of estrous cycles, and caspase 3 activity levels in three different group of (n=8) rats.

In our study, 30 pre-pubertal female sprague dawley were divided into groups of three with 10 rats, the mean weight of female SD rats aged 21 days was 40 g (32-49 gms) (**Figure** 1) was obtained from Aga Khan University, storage temperature (23±3°C) and humidity (54-66%) with 12 hours of circulation in dark light conditions and market food (2.45% carbs, 20.68% protein, 4.7% fat, 1.5% minerals, and 5.87 ash). Investigative practices were reviewed and approved through the Animal Welfare and Implementation Ethics Committee from Aga Khan University (84-ECACU-BBS-18) [17].

Body Weight Calculation

Body weight was evaluated from 8:00 a.m to 9:00 a.m throughout research work prior usage of DHEA.

Caspase 3 Estimation

Caspase 3 levels were measured by using ELISA (Cat. No. E1648Ra), which is a standardized 96 well plate sandwich ELISA procedure. It involves the preparation all reagents, standard solutions and samples. The entire procedure is done on room temperature. Initially, standard solutions and samples are added to their respective wells and incubated with anti-CASP3 antibody and streptavidin-horseradish peroxidase for 60 minutes at 37°C. During this time antigen-antibody complex are formed. Afterwards, the plate is washed five times to remove all unbounded antibodies and antigens. Finally, the substrate is added which results in coloration and stop solution is added to stop the reaction. Based on color intensity, optical density is determined through spectrophotometer at 450 nm, to quantify the antigen in the sample.

Statistical Analysis

Statistical analysis were performed using the IBM social science statistical package (IBM SPSS version 21; IBM Corp Inc.). Body weight was presented as mean±standard deviation. Independent sampling t-test was used to compare continuous variables, while Pearson's Chi-square test was used to find associations. Statistical significance was considered at $p<0.05$.

RESULTS

First, we examined whether PCOS model from pre-pubertal rats (21 days old) maintains high reproducibility of PCOS features. We investigated whether DHEA treatment altered the body weight of pre-pubertal rats. Since increase in body weight might be one of the major clinical features of PCOS patients, the increase in the ovarian and uterine weight was consistent with the increase in body weight (**Figure 2**).

The result shows that body weight of the rats increased from 88.83±1.84 (normal control rats) to 103.4±2.71 (PCO rats) in the time period of 30 days. In group III i.e. (treated rats) after vitamin D treatment their increased body weight back to 84.99±2.1 as shown in **Figure 2**. A significant increase in body weight was unclear from 21 to 28 days after DHEA injection compared with age-matched control rats. This result confirms that the intervention of Vitamin D in PCO condition can support in decreasing body weight, as shown in **Figure 2**.

Estimation of Caspase 3

Caspase 3 was used as an apoptotic marker to detect the increased activity of apoptosis in the rats of three different groups. The presence of apoptotic bodies in the PCOS group. Our results showed that caspase 3 levels in PCOS control group are significantly high than compare to normal control. We also find that after treatment of vitamin D in the rats for three weeks decreases the level of caspase 3 activity significantly as comparison to the PCOS control (**Figure 3**).

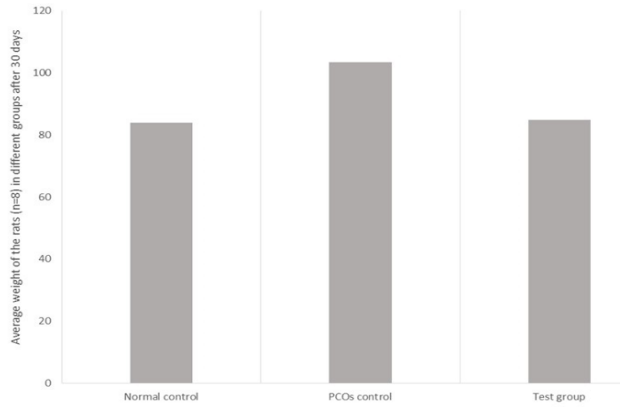

Figure 3. Caspase 3 activity recorded in three different group of rats

So, the levels of caspase 3 was decreased in vitamin D treatment group compared to the PCOS group.

DISCUSSION AND CONCLUSION

PCOS has a number of manifestations including oligomenorrhea, insulin resistance and hyperandrogenism and can eventually lead to metabolic disorders [2]. Research has confirmed this condition with endometrial hyperplasia and an increased risk of cancer [5]. Reproductive, morphological and anti- apoptotic marker were evaluated and to examine the effects of vitamin D over these findings in DHEA-treated female rats.

Meanwhile, obesity is closely linked to PCOS and can occur in up to 50% of cases. Anovulation occurs in overweight and obese women with PCOS. Therefore, greater weight gain 30 days after taking DHEA is due to the deposition of abdominal fat due to high androgen levels and an increase in the number of adipose tissues, especially in the body [18]. The rat model showed increased formation of follicular cysts in the ovaries, which occurred more frequently when DHEA treatment was started in a 21-day-old adolescent [19], which is consistent with our results. Another study concluded that vitamin D and calcium supplementation in women with PCOS increased body weight (BMI), follicle maturity, menstrual regularity, androgen-related symptoms, infertility, and resistance. Resistance has positive effects. In addition, vitamin D plays an important role in ovulation in women with PCOS.

Determining maximum reproductive levels of vitamin D and the need for vitamin D supplementation to reach these levels have important health implications for PCOS women [129. Among PCOS patients who received vitamin D, their weight decreased in 30 days. Administration of vitamin D (120 ng 1.25 (OH) 2D3/100) improved cycle characteristics, decreased body weight, and morphological characteristics in PCOS-induced animals. Although our study supports weight loss as reported by other studies. However, we agree that further research in a long cohort study is needed to reduce vitamin D weight. Several studies have investigated the efficacy of vitamin D supplements for PCOS women in which they have observed that PCOS women have hypovitaminosis D3, with growing evidence that vitamin D affects insulin and glucose metabolism [20].

PCOS is a disease known to be caused by changes in levels of cell growth and cell death factors and oxidative stress, leading to follicular arrest and immature follicles [21]. Thus, levels of interleukin 1b-converting enzyme-like proteases: caspases, in particular caspases 3, 7, and 9, which are important determinants of cellular activity, is studied extensively [21, 22]. Oxidative stress and cellular processes lead to the production of Reactive oxygen species (ROS) and/or reactive nitrogen species (RNS). Therefore, the human body has antioxidant processes in order to keep these oxidative species at minimal levels, preventing cellular damage [23], Where there is lack of balance between oxidative stress and antioxidant mechanisms, disease occurs due to mitochondrial mediated caspase pathway. This pathophysiological finding has been detected in the ovarian granulosa cells in PCOS [24]. Thus, programmed cell death in granulosa cells correlates with defective oocytes and poor IVF results in individuals with PCOS [25].

A variety of markers of apoptosis have been studies, one such marker is caspase 3. Caspase 3 is a pro-apoptotic protein, which plays a significant role during the process of programmed cell death. Thus, it is essential for chromatin clumping, breaking down of DNA, loss of cell membrane's integrity in addition to the production of final product of apoptosis: apoptotic bodies [6]. This apoptosis in ovarian granulosa cells result in oocytes having poor embryonic growth [26]. In PCOS, granulosa cells of ovarian cysts undergo apoptosis at later phases. Thus, caspase 3 levels are low earlier in order to avoid apoptosis and support the developing cysts, an anti-apoptotic protein, has shown to downregulate caspase 3 levels [27]. A research [28] demonstrated that levels of caspase 3 are augmented in the granulosa cells of PCOS patients as compared to non-diseased controls. According to the study in [29], ovarian cumulus cells had higher caspase 3 levels when compared to non-PCOS cells. Moreover, caspase-3 levels were positively associated with grade 2 embryos but negatively associated with grade 1 embryos, showing an inverse relation between caspase 3 levels and embryo's development.

Agreeing with prior studies, the researchers in [30] showed that pro apoptotic markers such as caspase 3, caspase 9, and bax were increased in granulosa cells of testosterone induced PCOS rats, while anti- apoptotic markers including Bcl-2 were decreased. Similarly, our results showed that caspase 3 levels in PCOS control group are significantly high as compared to normal controls.

In contrast to this, it was found that PCOS ovaries and follicles had low caspase 3 levels as compared to non-PCOS ovaries and proliferating follicles had high caspase 3, particularly in the granulose cells. On the other hand, ovarian cysts failed to show caspase 3 activity [27].

A study [22] demonstrated that in PCOS, serum has less caspase 9 levels, while caspase 3 and 7 failed to show a significant association between PCOS vs non-PCOS patients. In contrast to this, it was shown that PCOS ovaries have decreased levels of caspase 3, 8, and 9 [31]. Similarly, it was demonstrated that PCOS ovarian cells' caspase 3 level is lower as compared to normal cells [32]. Moreover, survivin is known to balance cell proliferation and cell death determinants, where it prevents apoptosis by decreasing levels of caspase 3 and 7, while ovarian hormones: LH and FSH, increase their levels [33-35].

In addition to caspases, bax and Bcl-2 are important in the transformation of normal follicles into immature cystic follicles. Bax is found to be raised in PCOS patients' ovary's epithelium, follicles and cysts [27]. It was demonstrated increased staining for caspase 3 in uterine tissue samples of PCOS patients, which decreased to lower levels post treatment with vitamin D, although not significantly [10]. Similarly, our study showed that after treatment with vitamin D for three weeks, the level of caspase 3 activity decreased significantly as compared to the PCOS control vitamin D works at the cellular level to prevent oxidative DNA damage [36]; thus, has been postulated as a possible therapy for PCOS as it decreases inflammatory mediators, increases insulin sensitivity and alters calcium homeostasis [37]. Moreover, it modulates the immune system by playing a role in the functions of inflammatory cells including lymphocytes, monocytes, and phagocytic cells [38]. Apart from having a protective role for apoptosis in PCOS, vitamin D treatment has also shown to improve rickets [39], migraine [40], copper tunnel syndrome [41], and stroke [42]. Vitamin D decreases proliferation of endometrium, decreasing the risk of endometrial carcinoma. Moreover, through its effect on the endometrium, embryonic implantation and pregnancy outcomes can be improved. Thus, concluding from these studies, vitamin D maybe a suitable supplement to prevent associated disorders in PCOS. However, in order to study the effects in extensive detail, a longer duration of vitamin D treatment and further studies are warranted.

The dose of vitamin D in human studies will differ from that in rats. Rodent models have helped us to understand the pathogenesis of PCOS and to develop possible therapies for PCOS. The results confirm the effect of vitamin D therapy on metabolic and reproductive properties in women with PCOS. It is suggested to evaluate the results by providing more therapy for further research. Therefore, it could be an excellent model for further development of important new therapeutic procedures and language for PCOS management.

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Data sharing statement: Data supporting the findings and conclusions are available upon request from the corresponding author.

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