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The consequences of caffeine exposure on the growth of the embryonic brain during different stages of chick embryo development

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Abstract

Caffeine is a natural ingredient found in coffee, tea and cocoa, it is also added to some soft drinks, energy drinks, and to some over a counter medication, pregnant women consume caffeine during pregnancy, which leads to a teratogenic effect on embryos, because it passes easily from a mother's blood to embryos through a placenta. We measured the effect of caffeine on a morphological and histological abnormalities in a brain of chicken embryos, where embryos were incubated after sterilization of eggs in an incubator at a temperature 38°C, and embryos were injected with caffeine at concentrations 2 and 3 mg/ml at stage HH8, which a second day of incubation. Embryos were collected at stages HH17, HH21, HH29 and general morphological changes were observed and histological alterations in brain development were examined. The results showed abnormalities in the brain, where a small size of the brain and sometimes a fusion of the brain in the cranium.

Keywords *Caffeine, Brain, Chicken embryos development.*

1. Introduction

Research indicates that approximately 95% of pregnant women consume caffeine, either as a beverage or in conjunction with medications. The molecular structure of caffeine bears a resemblance to components of DNA, prompting concerns among researchers regarding its potential teratogenic and mutagenic properties [1], [2]. Caffeine is capable of crossing the placental barrier, transferring from the mother to the developing embryo[3]. The primary enzyme responsible for caffeine metabolism, cytochrome P450 1A2 (CYP1A2), is absent in both the

placenta and the embryo, suggesting that maternal caffeine consumption during pregnancy can significantly influence fetal development based on the levels of caffeine exposure [4].The overexposure of the fetus to caffeine can cause an increase in catecholamine levels, leading to vasoconstriction and hypoxia in the fetal-placental circulation. This, in turn, can impact the growth and development of the fetus [5]. Notably, caffeine exhibits an extended half-life during pregnancy, as it cannot be metabolized by the embryo or the placenta [6]. Moreover, earlier investigations have associated caffeine consumption in pregnant women with an elevated risk of miscarriage. The studies reveal that those who underwent late miscarriages or stillbirths had significantly greater caffeine intake [7]. Therfore this study was performed using a chick embryo model to assess the influence of caffeine introduced in the early stages of embryonic development on the morphological and histological characteristics observed at distinct developmental phases.

2. Materials and Methods:

Experimental design

Fertilized chicken eggs *Gallus gallus*, were obtained from local farm in eastern Libya. One hundred and eighty eggs were divided into four groups of 45 eggs in each group. The first group was the control group, which was left without any treatment, second group was injected with distilled water which is injected with distal water, and third group injected with 2mg of caffeine, fourth group injected with 3mg caffeine. They were then incubated on their sides at a temperature of 38 °C and a humidity level of 80% for 26-29 hours which is HH8 staged in accordance with the definitions provided by Hamburger and Hamilton (HH) [8]. The embryos were collected at different developing stages HH17, HH21and HH29 and fixed in 10 % formalin and kept for morphological and histological analysis.

Egges injection

After 26-29 hours post incubation, the eggs were exposed to room temperature. Subsequently, a small amount, about 1.5 to 2 ml of albumin was withdrawn from the small hole created by needle in blunt end to facilitate the embryo's movement towards the eggshell. Following this, caffeine 2mg/ ml solution was injected utilizing a needle with an outer diameter of 0.60 MM (size23G x1.1). Post-injection, the holes created in the eggs were sealed with tape, and the eggs were reintroduced into the incubator for further development.

Embryos Collections and fixation:

Following an incubation duration of 2.5, 3.5, and 6.5 days, the eggs were removed from the incubator with great care. The upper shell was then opened with precision using micro scissors. The embryos were delicately placed into a petri dish containing phosphate buffer solution (PBS) . The extra embryonic membranes were removed with the aid of micro scissors. Morphological evaluations and photography of the embryos were performed using a dissecting optical microscope fitted with a fixed canon IXUS 125 HS digital camera 16.1 mega pixels. The average embryos surface aria was measured from photos using soft wear "Image J". The embryos were subsequently embedded in paraffin wax, and sections of 5 microns in thickness were carefully prepared and stained with hematoxylin-eosin for further analysis.

Preparation of histological paraffin embedding sections

The embryos were dehydrated through a series of increasing ethanol concentrations, infiltrated with xylene, and subsequently embedded in paraffin. Thin sections of 5 µm were then cut and processed according to standard methods for hematoxylin and eosin staining. Tissues were examined under a light microscope, and photographed with

3. Results and Analysis

There is a link between caffeine consumption and defects in a nervous system due to excessive maternal caffeine intake. In this study, early chick embryos were used as a model to evaluate these effects with different concentrations of caffeine, as many abnormal phenotypic defects appeared, and the following will explain an effect of each concentration on different stages of chicken embryos:

Embryos treated with 2 mg/ml:

A control group in this group was 100% alive, with fertility ranging from 90 to 100 %, and a percentage of abnormalities was 0% at all stages HH17, HH 21 and HH29, as shown in figure 1. A same thing happens to embryos that have been injected with distilled water.
Embryos injected with 2 mg/ml had a survival rate of 90%, a death rate of 10%, a 100% abnormality rate, and a fertility rate of 90% for a group collected at stage HH17. A survival rate for stage HH21 embryos was 90%, death was 10%, abnormalities were 100%, and fertility was 100%. A percentage of embryos gathered at stage HH29 that survived was 80%, death was 20%, and abnormality was 100%, whereas a percentage of fertility was 100%.

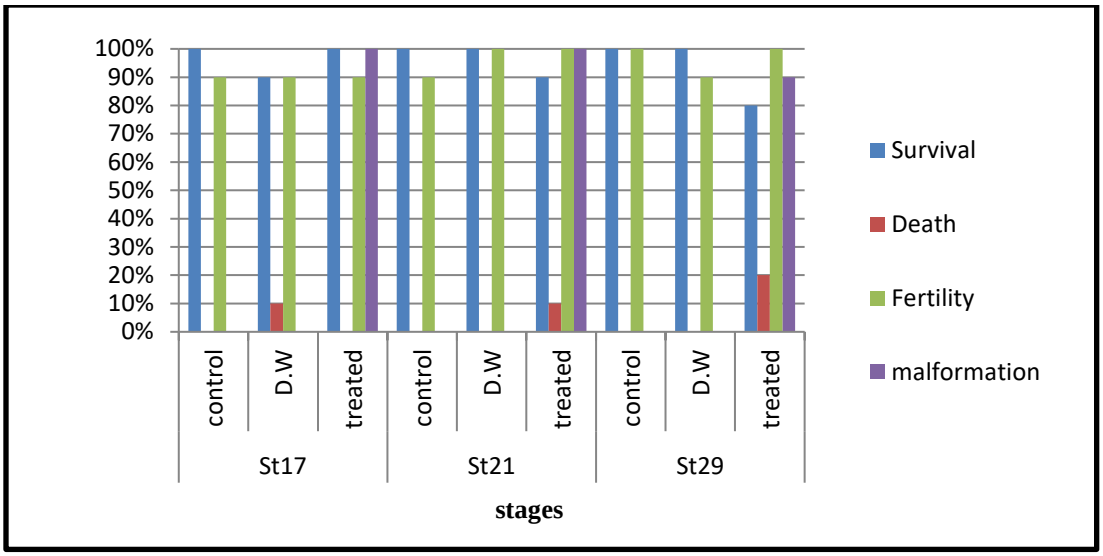

Figure 1:Histogram showing the Percentage of survival ,death, fertility, and malformation of embryos injected with 2 mg/ml(caffeine) at HH17,HH21,HH29.

The graph chart illustrated in figure 2, showed the total embryo surface area for various stages of embryos HH17, HH21 and HH29, in comparison to control groups, distilled water, and embryos treated with 2 mg/ml caffeine.

It is obvious that there were no differences in a mean for overall surface area in mm between a control and distilled water groups of chicken embryos. In contrast, when compared to a control group and distilled water, a mean for entire surface area in mm decreased for embryos treated with 2 mg\ml.

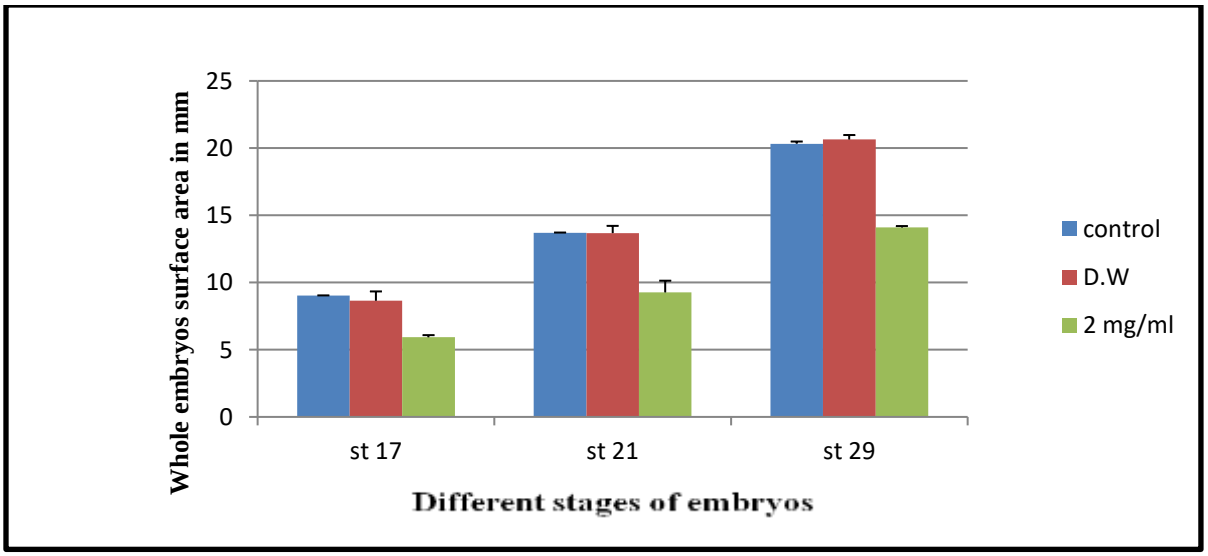

Figure 2: Histogram showed a whole embryos surface area in mm. between control, distilled water and concentration 2 mg/ml of caffeine. Error bar presented by standard deviation.

The impact of caffeine on chicken embryos at different developmental stages is depicted in figure 3 below. The comparison among a control group, a group treated with distilled water, and a group treated with caffeine at stage HH17, indicated that the caffeine treated group exhibited significant differences from both the control group and the distilled water group. Within both figures, A1 denotes the control group, while B2 represents the distilled water group, where embryos displayed normal organ growth, extended somites, unpigmented eyes, complete brain development, and a normal heart. Conversely, embryos treated with caffeine (C1) showed signs of developmental delay, deficits, reduced weight, microcephaly, craniofacial abnormalities, and abnormal heart growth. At stage HH21, both the control and distilled water groups (A2 and B2 respectively) showed regular development with normally developing organs, appropriately divided head structures, eye pigmentation, regular heart development, tail orientation towards the head, wide limb buds, and correctly curved trunks. In contrast, the caffeine group (C2) exhibited reduced growth, smaller size, decreased weight, microcephaly, heart irregularities, absence of eyes in some embryos, abnormal tail curvature, trunk shape distortion, and absence of limbs. Moving on to stage HH29, it was observed that the brains of the control and distilled water groups developed normally, along with the growth of front and hind limbs, eye pigmentation, and the presence of three fingers, groove lines between fingers, and the beginning of beak formation. A3 represents the control group, while B3 denotes the distilled water group. In comparison, the caffeine-treated group (C3) displayed smaller embryos, heart deformities, and brain distortions..

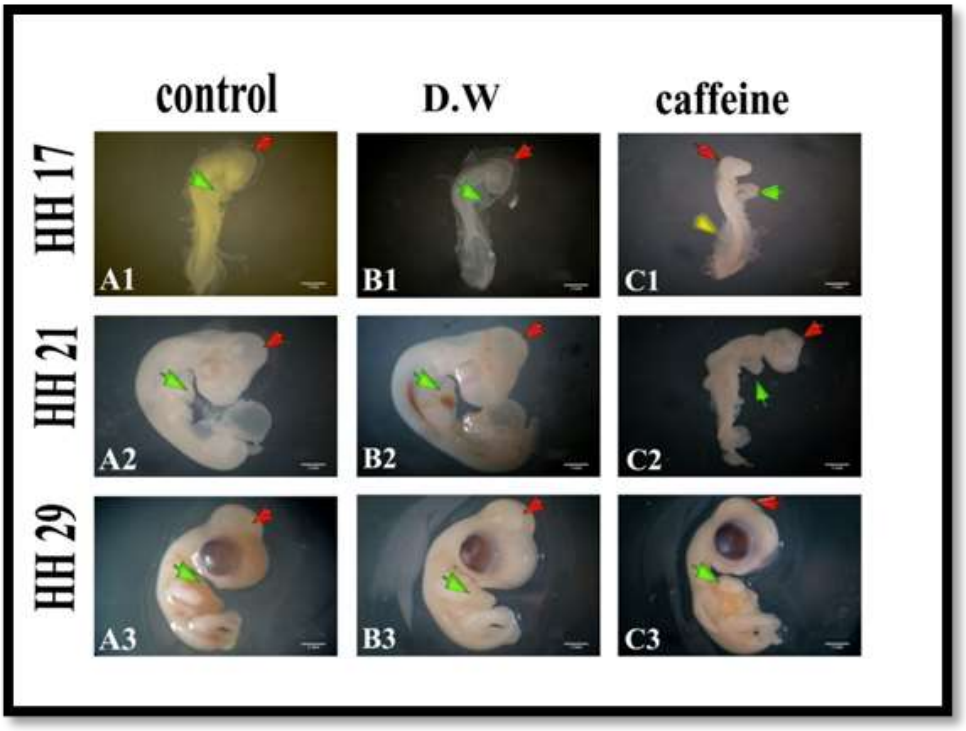

Figure 3: Showed an effect of caffeine at a concentration of 2 mg / ml on embryo growth at different stages. Control A1 stage HH17, A2 stage HH21 and A3 stage HH29, distilled water B1 HH17, B2 HH21 and B3 HH29, treated by caffeine C1 stage HH17, C2 stage HH21 and C3 stage HH29. Red arrows point to a brain, green arrows indicate a heart and yellow arrow indicate a trunk.

Embryos treated with 3 mg/ml:

Control group showed 100% alive, with fertility ranging from 90% to 100%, and a percentage of abnormalities were 0% at all stages HH17, HH21 and HH29, as shown in figure 4. A same holds true for embryos that have been injected with distilled water. For embryos injected with 3 mg/ml, a group collected at stage 17 had a 100% survival rate, 0% mortality, 100% abnormalities, and 100% fertility. Embryos collected at stage HH21 had an 80% survival rate, a 20% mortality rate, a 90% abnormality rate, and 100% fertility. A percentage of embryos that survived after being retrieved at stage HH29 were 90%, death was 10%, and abnormality was 90%, while a percentage of fertility was 100% as indicated in figure 4.

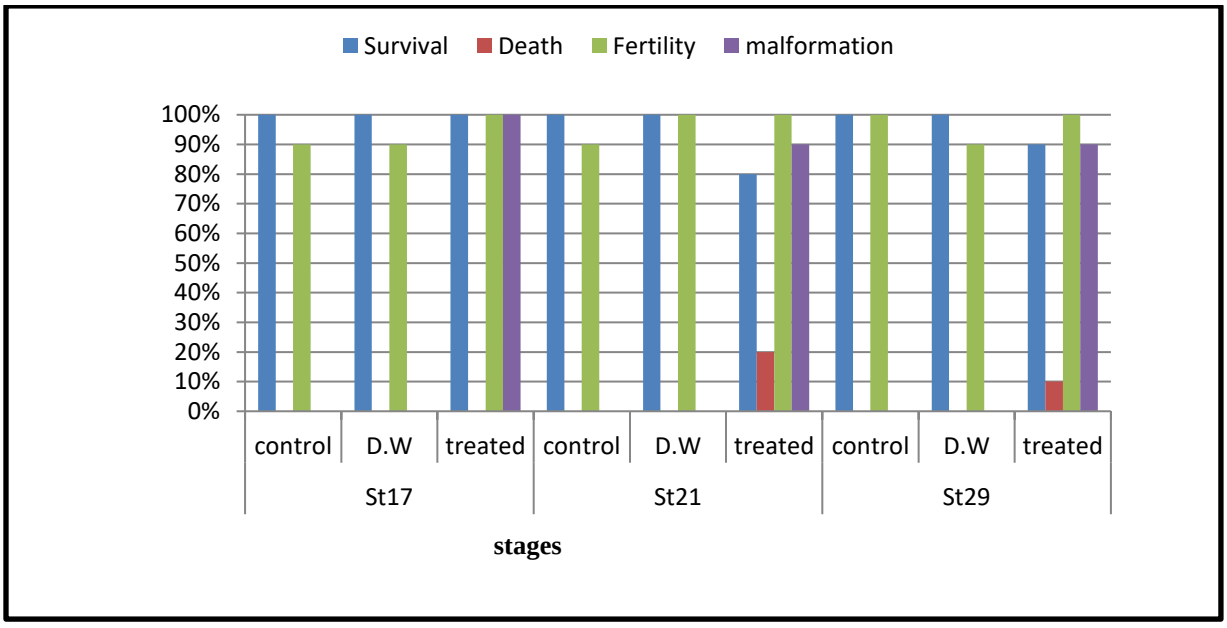

Figure 4: Histogram showed a percentage of survival, death, fertility, and malformation of embryos injected with 3 mg/ml of caffeine at stages HH17, HH21 and HH29.

The graph chart illustrates figure 5, a total embryo surface area for various stages of embryos HH17, HH21and HH29 in comparison to control groups, distilled water, and embryos treated with 3 mg/ml caffeine. It is obvious from a data in figure 14 that there were no differences in a mean for overall surface area in mm between a control and distilled water groups of chicken embryos. In contrast, when compared to a control group and distilled water, a mean for entire surface area in mm decreased for embryos treated with 3 mg\ml.

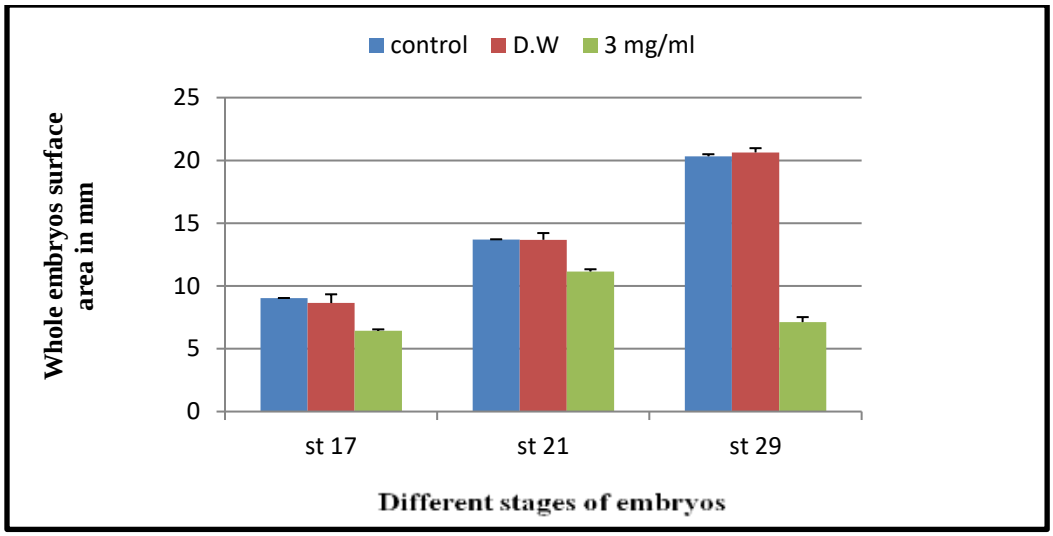

Figure 5: Histogram showed a whole embryos surface area in mm. between control, distilled water and concentration 3 mg/ml of caffeine. Error bar presented by standard deviation

A diagram shown in Figure 6 below illustrates the impact of caffeine on chicken embryos at various developmental stages. Upon examining the differences between a control group, a group given distilled water, and a group treated with caffeine at stage HH17, it was observed that in both scenarios, where A1 denotes the control and B2 denotes distilled water, there were no discernible differences as the embryos exhibited normal organ growth with extended somites forming a tail, unpigmented eyes, a complete brain, and a normal heart. However, embryos treated with caffeine (C1) experienced growth retardation, lower embryo weight in comparison to the control and distilled water groups, microcephaly, craniofacial abnormalities, and abnormal heart development, with some embryos displaying significantly enlarged hearts compared to the typical heart form.

At stage HH21, both the control and distilled water groups (A2 representing the control, B2 representing distilled water) demonstrated normal embryo development, with organs developing properly, a naturally divided head, pigmentation observed in the eyes, normal heart development, a headward tail, wide limb buds, and a naturally curved trunk. Conversely, the caffeine-treated group (C2) exhibited reduced growth, smaller size, lower weight compared to the control and distilled water groups, microcephaly, heart deformities, absence of eye pigmentation, abnormal trunk and tail curvature, and micromelia. Moving on to stage HH29, normal growth was noted in both the control and distilled water groups (A3 representing the control group, B3 representing the distilled water group), with typical brain development, eye growth, eye pigmentation, normal front and hind limb formation, embryos with three fingers and groove marks between them, and the beginning of beak formation. However, embryos treated with caffeine (C3) displayed smaller size, decreased growth compared to the control and distilled water groups, microcephaly, cranial deformities, fusion of the head with the brain resulting in a shapeless mass, absence of normal eye growth, occasional complete eye absence, heart distortion, absence of limbs (micromelia), and noticeable trunk distortion.

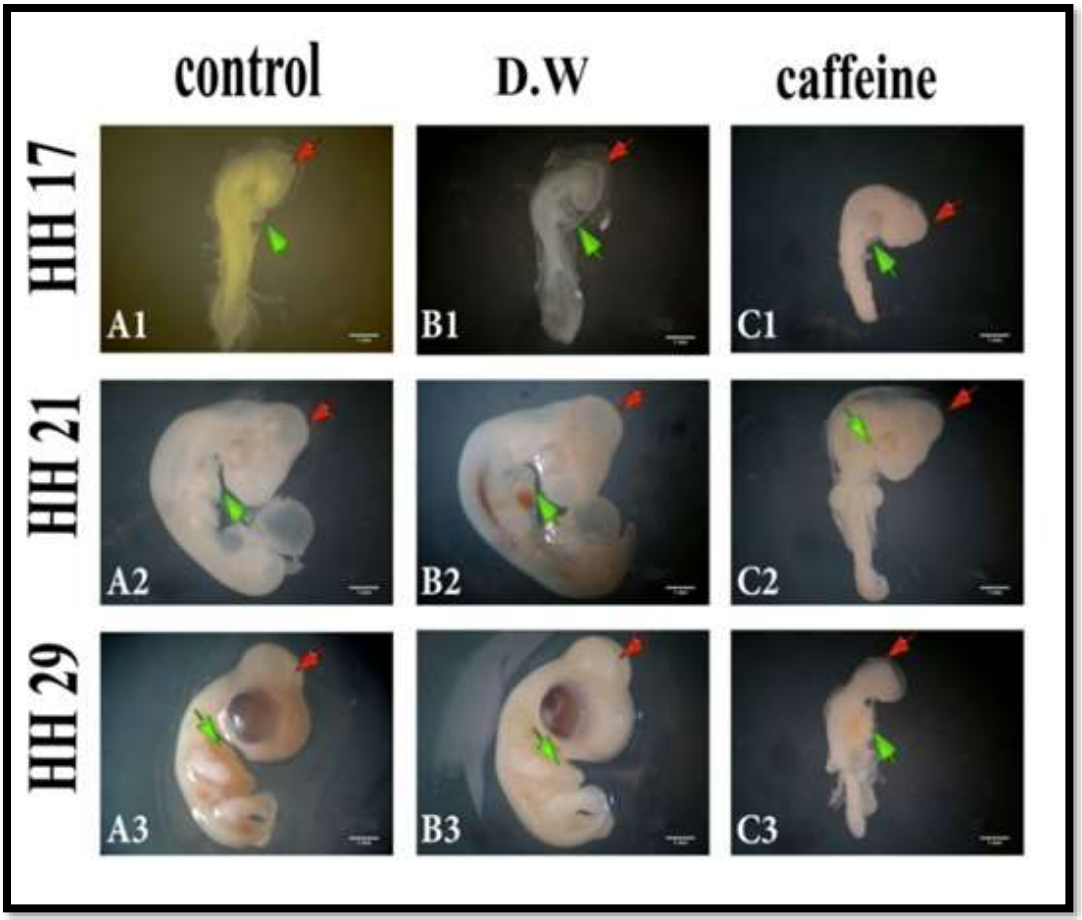

Figure 6: Showed an effect of caffeine at a concentration of 3 mg / ml on embryo growth at different stages. Control A1 HH17, A2 HH21 and A3 HH29, distilled water B1 HH17, B2 HH21 and B3 HH29, treated by caffeine C1 HH17, C2 HH21 and C3 HH29. Red arrows point to a brain and green arrows indicate a heart.

Histological section results

Figure 7 below cross section at diencephalon region, showed an effect of caffeine on chick embryos at stage HH21. It observed that there is a difference between a control group and a group treated with caffeine. Figure 7-A' represents a control, a normal brain layer, vernacular, subventricular, intermediate, cortical and marginal layers. While in figure 7-B' which represents embryos treated with 2 mg/ml, the brain layers ware thicker and more blood vessels in the marginal layer and numbers of stained nuclei was increased, cells were loose. Figure 7-C', which represents embryos treated with 3 mg/ml, were noted thickening was observed in a brain layer and also cell shapes appear different from control which look dendritic were shorter.

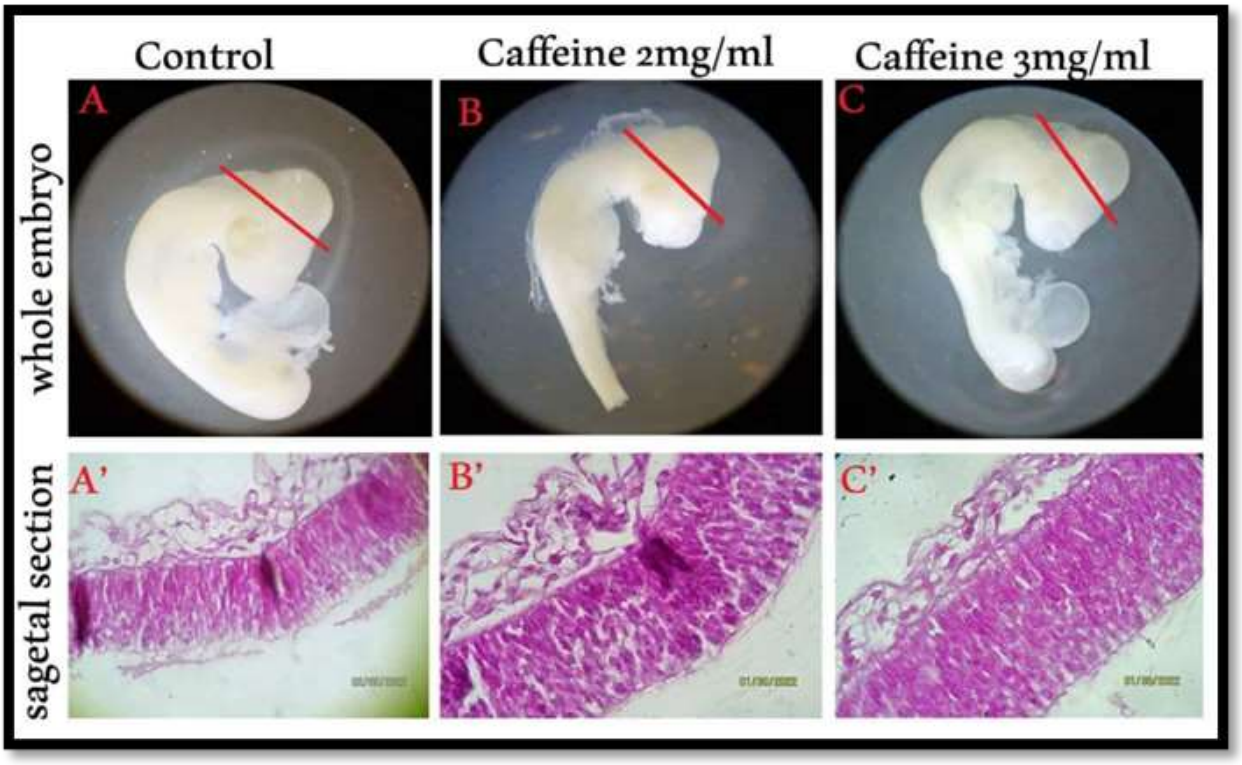

Figure 7: A cross section in a brain region. Where A represents a control, B represents an embryo treated with 2 mg/ml of caffeine, C represents an embryo treated with 3 mg/ml of caffeine.

Discussion:

The detrimental effects of alcohol and tobacco are consistently highlighted in the public sphere. In contrast, the potential hazards linked to caffeine, a substance widely ingested in our daily diet, are not given as much prominence. Consequently, there is a prevailing belief that caffeine poses a significantly lower risk compared to alcohol or tobacco. Recent studies have focused on the consumption of caffeine, particularly in the early stages of pregnancy. This current research endeavors to contribute to the existing literature by underscoring the teratogenic effects of caffeine. Caffeine is prohibited or advised in low doses during pregnancy due to their ability to cross a placental membrane and accumulate in an embryo's body. In several studies caffeine consumption has been linked to lower rates of fertilization, embryonic implantation, changes in placental structure, low fetal weight and an increased risk of intrauterine growth restriction (IUGR) [9],[10] Chick embryos have been used, because an early embryonic development of chicken is very similar to that of humans. The present study utilized morphological and histological evaluations to explore the potential risks linked to maternal caffeine consumption on the neurological and cardiac development of the fetus. The results identified various morphological abnormalities in the heart, brain, limb buds, trunk, and other organs, as demonstrated in Figures 3 and 6.

At a concentration of 2 mg/ml, a death rate of embryos was 20%, while a percentage of malformations ranged between 90% - 100%, as shown in figure 1. At a concentration of 3 mg/ml, a rate of fetal death was 10%, while a percentage of malformations ranged between 80% - 100%,

as shown in figure 4. It was clear through results of this research, exposure to caffeine alters brain development, small brain size and in later stages showed changes in brain tissue as showed figure 7 and cell proliferation, which is consisted with study revealed that, caffeine can cause over expression of PAX6 [11] , which involved in differention in bran cells. Another study found that , caffeine consumption during critical periods of pregnancy can produce epigenetic changes in a developing embryo or even germ cells, which can lead to adult-onset illnesses in future generations [12]. Most of the abnormalities observed in a specific brain region can be attributed to the potential impact of Caffeine on the development of the neural tube. This can result in modifications to the expression of morphogens that govern the pattern formation of the neural tube, such as Wnt and BMP situated in the dorsal region, leading to changes in Pax7 and Pax6. in addition Shh serves as a significant morphogenetic factor believed to play a crucial role in regulating neural and oligodendroglial progenitor populations throughout the developmental process.[13], the presence of caffeine may influence the expression of Shh in the ventral region of the neural tube, subsequently causing alterations in Nkx6.1 expression. Furthermore, caffeine can impede the function of adenosine receptors, thereby sustaining an appropriate oxygen level in embryos. Consequently, caffeine disrupts the normal oxygenation process within cells. Additionally, due to the structural resemblance between caffeine and adenosine molecules, which is pertinent to DNA methylation, caffeine has the potential to induce mutations in the DNA methylation process. Numerous research studies have corroborated that exposure to caffeine can potentially impact the neural fold closure process [14], [15], [16].

5. Conclusion

In this research we used chicken embryos to study an effect of caffeine on the brain of embryos in stages HH17, HH21 and HH29. Embryos treated with caffeine showed various abnormalities such as small head size, abnormalities in a brain, these abnormalities are represented in a microcephaly of the brain with a cranium and its absence in some cases, when compared with control. Thus, caffeine consumption during the stages of embryo development could heighten the risk of birth defects. This study offers a preliminary understanding of the implications of caffeine intake during pregnancy; however, additional in-depth research is necessary to explore this area further.

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