wjpmr, 2024, 10(2), 161-168

SJIF Impact Factor: 5.922

WORLD JOURNAL OF PHARMACEUTICAL AND MEDICAL RESEARCH

www.wjpmr.com

Research Article

ISSN 2455-3301

WJPMR

TOXICOLOGICAL EFFECTS OF CAPSAICIN, CAFFEINE, AND NICOTINE ON THE DEVELOPMENT OF CHICK EMBRYOS

Bolinjkar A. N., Singh Ayushi* and Talpade M. B.

C 203 Building no. 7, Ratan Nagar Lane, Four Bunglows, Andheri West C203 Building no.7
Mumbai Maharashtra 400053.

***Corresponding Author: Singh Ayushi**

SVKMs' Mithibai College of Arts, Chauhan Institute of Science & Amrutben Jivanlal College of Commerce and Economics (Autonomous)

Article Received on 20/12/2023 **Article Revised on 10/01/2024** **Article Accepted on 30/01/2024**

ABSTRACT

The environment encompasses a diverse array of deleterious compounds which is crucial to consider the potential on humans to elicit toxicological responses in other organisms. Notable examples include caffeine, capsaicin, and nicotine which are consumed all around the world and are part of our everyday lives. This study aims to explore the toxicity and teratogenicity of these toxins on 72-hour-old chick embryos. Three concentrations (5%, 10%, and 100%) of the isolated toxins were investigated. The findings indicate a significant impairment in the developmental capacity of treated embryos. Caffeine and capsaicin resulted in reduced sizes and morphological alterations in the embryos. Nicotine exhibited detrimental effects on eggs, leading to embryo demise. Capsaicin appeared to impact both neural and internal organ development. A substantial hindrance in growth is characterized by caffeine's effects. In conclusion, caffeine exposure can affect the closure of neural folds, Capsaicin can lead to retarded growth, and nicotine toxicity can affect brain development. However, the exact mechanism of these effects requires further investigation.

KEYWORDS: Toxicity, Chick embryo development, Capsaicin, Caffeine, Nicotine.

INTRODUCTION

The chili peppers contain capsaicin, which gives them their characteristic strong flavor. Capsaicin is unique among naturally occurring irritating chemicals (Sharma *et al*., 2013). It is a waxy, colorless material that is hydrophobic. Pure capsaicin irritates whatever surface it comes into touch with. Because this receptor is located on critical sensory afferents, the use of capsaicin to selectively activate pain afferents has been investigated in animal and human models for a variety of applications. Its ability to bind to taste and vanilloid receptors in the mouth, leading to a burning sensation, renders capsaicin toxic to numerous mammals (Talk of the Nation, 2008). However, birds remain unaffected by capsaicin as they enable seeds to pass through undisturbed, in contrast to mammals whose teeth could potentially destroy them. (O'Neil *et al*., 2012). Understanding the activities of capsaicin resulted in the identification of its receptor, transient receptor potential vanilloid subfamily member 1. According to Johnson and Wilbur (2007), it has an LD_{50} of 47.2 mg/kg in mice. However, the toxicity in humans has not been established. While capsaicin's impact on isolated neurons has been investigated, there is a lack of in-depth exploration into its effects at various doses on the overall development of chick embryos (Akiro *et al*., 1987).

Caffeine is the most popular psychoactive substance in the world, more than 80% of persons routinely drink caffeine. (*Caffeine*, n.d.) It can be present in common consumer beverages and food, including coffee, tea, soft drinks, cocoa, chocolate, and several pharmaceuticals. (Li *et al*., 2012) Caffeine stimulates the brain, lifts the mood, and delays exhaustion. It also improves performance on easy intellectual tasks and physical work that requires endurance but not fine motor coordination. (*Caffeine*, n.d., *loc. cit*.) Neurodevelopment is governed not only by spatiotemporal gene expression but also by the embryo's external environment, which has the greatest influence during cranial neural crest cell delamination. (Ma *et al*., 2012b) Caffeine also has observed effects on neurotransmitters as well as the inability to get metabolized in the embryonic brain (Li, *loc. cit*.). Studies have shown a relationship between coffee use and neural tube abnormalities (Schmidt *et al*., 2009, Schmidt *et al*., 2010, Ma, *loc. cit*.). Previous research (Ma, *loc. cit*., 2012, Kimmel *et al*., 1984, Galli *et al*., 1975, Tanaka *et al*., 1987) has demonstrated that caffeine can enter the embryo from the external environment and accumulate in the fetal brain. As a result, it is possible that maternal caffeine consumption can disturb normal neurodevelopmental processes, so acting as a teratogen. This information raises concerns,

especially since pregnant mothers often consume coffee and other caffeinated beverages, potentially affecting the development of the fetus as mentioned by Fakhr El-Din M. Lashein and Amin Abdou Seleem, 2012. It is possible that maternal caffeine consumption can impair normal neurodevelopmental processes, so acting as a teratogen. (Ma, *loc. cit*.)

Nicotine accelerates message transmission between the brain and body. Cigarettes, cigars, pipe tobacco, chewing tobacco, wet and dry snuff, and dried tobacco leaves all contain nicotine. Electronic cigarettes (sometimes called vapes) do not include dried tobacco leaves, although they may contain nicotine. (*Nicotine - Alcohol and Drug Foundation*, n.d., 2024) Notably, nicotine is a potent stimulant of the parasympathetic nervous system, as indicated by Haas and Kubler in 1997, leading to sensations of tranquility, alertness, and overall relaxation. (Lagrue *et al*., 2001). Given that nicotine is derived from plants in the Nightshade family, which also includes the notorious Deadly Nightshade (*Attropa belladona*), nicotine's status as a lethal toxin is not surprising (Brandon *et al*., 2015). It has an LD_{50} of 50 mg/kg for rats (In chem, 2012) and 30–60 mg (0.5–1.0 mg/kg) in humans (Okamoto, *et. al*., 1994). Despite the absence of conclusive evidence categorizing nicotine as a carcinogen, it is frequently regarded as a promoter. (Cardinale *et. al*., 2012). This makes it the most studied drug.

Common knowledge emphasizes that no amount of nicotine is considered safe during pregnancy. The Surgeon General in the United States in 2014 highlighted that nicotine exposure can impact brain development, lead to fetal death (Wilder *et al*., 2016), and increase the risk of various health conditions in the child, including obesity, hypertension, neurobehavioral defects, respiratory dysfunction, and infertility (Schraufnagel *et al*., 2014). Nicotine rapidly crosses the placenta, and levels in fetal serum and amniotic fluid are higher than those in maternal serum. (Suter & Aagaard, 2020). Moreover, nicotine has been found to influence the development of cardiovascular systems and the liver (Rosenbruch *et al*., 1993). Extensive studies, such as those by England *et al*. in 2017, have explored the effects of nicotine on the brain development of different fetuses.

The chick embryo has revolutionized toxicological studies in recent years. Chick embryos prove to be an excellent alternative to live animal subjects, minimizing the loss of life. Given the similarity in embryos among chordates, the impact of toxins on chick embryos is likely indicative of effects on other animals, as noted by (Gilbert, *loc. cit*.). This extends to humans as well. Chick embryos are relatively easy to care for and develop in a laboratory environment, making observations straightforward, especially when there are alterations in developmental characteristics.

MATERIALS AND METHODS

Capsaicin- Capsaicin was extracted using a Soxhlet Extractor, with methanol as solvent (Ashwini *et al*., 2015). Thai bird eye chilies (*Capsicum annuum*) were bought from the nearest supermarket. The chilies were de-stemmed and subsequently dried in an oven for two days at 90°C to remove any remaining moisture. After, the de-stemmed and oven-drying, chilies were finely ground into a powder which was then combined with 300 mL of methanol using a Soxhlet extractor, resulting in the extraction of a viscous, oily substance. This extracted form of capsaicin was further used for analysis.

Caffeine- Caffeine was extracted from Instant coffee readily available in the market using a Soxhlet extractor and separated using Chloroform (Schaber *et al*., 2012). The resulting dark brown liquid was extracted and transferred into a beaker, where it was left to reach room temperature. Subsequently, the Methanol-Caffeine solution was combined with 10g of Magnesium Oxide dissolved in 70 mL of distilled water, as described by Tumimbang *et al*. in 2014. The heated solution was then mixed with concentrated sulfuric acid (2M) and was further evaporated. The mixture was then allowed to cool at room temperature. After cooling the mixture was treated with chloroform. To eliminate the distinct yellow color, 2mL of sodium hydroxide and 2mL of distilled water were added. The evaporation of the chloroform yielded a white powder, and this unprocessed form of caffeine was used for the injection.

Nicotine- Nicotine was extracted using commercially available chewing tobacco, Sodium hydroxide, and diethyl ether. The method employs the utilization of a separating funnel (Pavia *et. al*., 1976). Tobacco leaves, obtained from a street vendor in 10 packs of 8g each, were used for the experiment. 20g of the leaves were weighed, which was dissolved in 200 mL of 10% sodium hydroxide. This process was repeated four times and each suspension of nicotine and sodium hydroxide was then left overnight in a 250 mL conical flask. After 24 hours, leaves were filtered with muslin cloth and soaked in water for another 30mins and later, sodium hydroxide was mixed with the water. Using a vacuum pump, a Buchner Funnel, and Glass Wool, the residual liquid was drawn from the leaves and allowed to sit for 30 minutes. The supernatant was then decanted into a separate flask and 200mL of the dark brown liquid was then poured with 20mL of Diethyl Ether.

The separation takes place as Nicotine is more soluble in Ether as compared to Sodium Hydroxide or water (Gonçalves and Minas da Piedade, 2012). The supernatant layer was removed, dried with anhydrous potassium carbonate, and filtered. The solution was then placed in a beaker, covered with perforated tin foil, and allowed to rest overnight. Once the ether is evaporated the dark yellow, oily residue left behind is nicotine.

Inoculation

Each of the prepared samples was poured into sterile plastic containers. The samples were to be used in three varying concentrations. They are as follows:

- Pure toxin, i.e., 1X or 100%
- One part toxin plus ten parts Distilled water, i.e., 0.1X or 10%
- One part toxin plus twenty parts Distilled water, i.e., 0.05X or 5%

Nicotine and Capsaicin were directly injected into the chick embryo. The immobilization of caffeine involved using a single drop of distilled water with a substantial amount of caffeine powder to maximize the concentration.

The 24-hour fertilized eggs were collected from Aarey Colony in Mumbai and were kept in an incubator for 72 hours at a temperature of 37 °C. A modified version of the window approach was then used to inoculate the eggs (Korn and Cramer, 2007).

The embryo was spotted in the eggs using the candling method. Once the embryo was spotted, a small window of 1cm X 0.5cm was created using a No. 11 sterile surgical scalpel. The shell over the window was carefully removed and the inner membrane was delicately sliced with the scalpel. Samples were now injected into the embryo using a sterile syringe. The removed shell was accurately placed in the original place and secured using scotch tape. The embryos were then incubated at 37℃ for 144 hours. For each concentration of each toxin, three eggs were inoculated. The eggs were opened and closed without any injections, aiming to eliminate any errors in technique serving as negative control. After 144 hours, the embryos were gently removed, washed in distilled water, and placed on a sterile slide in a petri dish for preliminary observations.

The developed embryos were subsequently transferred to sterile glass bottles containing the 10% neutral buffered formalin. These bottles were labeled with their respective concentrations and stored in the refrigerator for further analysis.

RESULTS AND DISCUSSION

The embryos were observed using a Single Lens Dissecting Microscope with magnification of 10X. The results were as follows:

Control (Negative and Positive)

The embryos from both control groups exhibited normal development as shown in Figures 1.1 and 1.2. Following the accepted model of chick embryological development, as outlined by Navis and Adam in 1951, the formation of the eye, brain, limbs, and other structures occurred simultaneously.

Image 1.1: 144-hour-old Chick Embryo in the Egg (Healthy).

Image 1.2: 144-hour-old Embryo in a petri dish (Healthy).

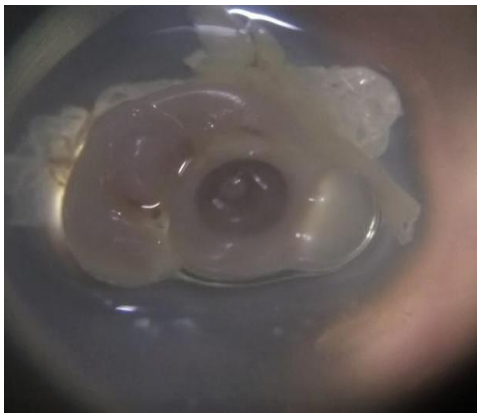

Image 1.3: Negative Control Chick Embryo.

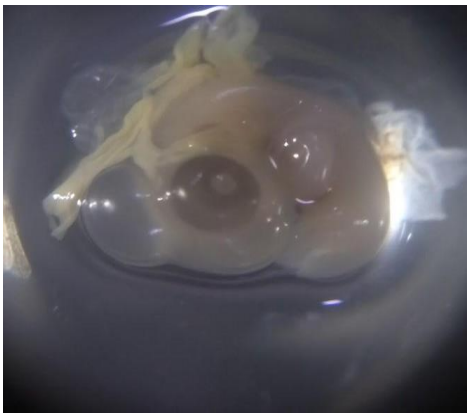

Image 1.4: Positive Control Chick Embryo.

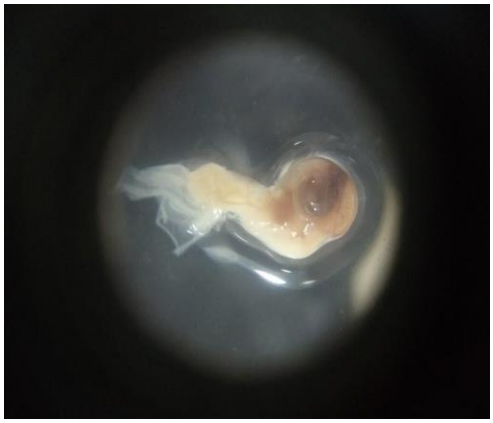

Image 1.5: Embryo inoculated with 5% Capsaicin.

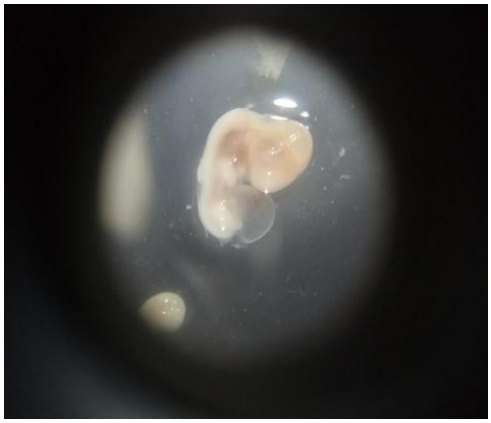

Image 1.6: Embryo inoculated with 10% Capsaicin.

Capsaicin

Death was observed at all three concentrations of capsaicin. Blood vessels were well developed but the neural and organ development was hampered showing a limited amount of growth in the embryos.

1. **5%:** Growth was slowed in embryos injected with 0.05X capsaicin. The eye and brain were only partially grown, and a reddish material had been deposited in the head and abdomen showing the presence of capsaicin. Limbs and skeletal structures were smaller than in the positive control group, and they appeared to be underdeveloped
2. **10%:** The chick embryo injected with 0.1X capsaicin showed considerably slower growth. The eyes, brain, and body appeared smaller, and the eye was not prominent through the membrane making it less noticeable. Abnormal body curling was noticed, and the reddish deposits of capsaicin were more visible in the abdominal region than in the head.
3. **100%:** The embryos injected with 1X were the most severely damaged and had the smallest size as compared to other concentrations. There was no organ differentiation, and the brain, eyes, organs, and other tissues did not appear to have evolved. The shape showed modest growth, and a noticeable reddish color was seen throughout the body.

The reddish deposition in the abdominal region of the embryo was observed suggesting that the capsaicin was directly absorbed by the embryo but possibly metabolized improperly or incompletely. The Embryo with 100% concentration of capsaicin showed no differentiation and retarded growth. This could be attributed to the hypothesis that capsaicin metabolism might not be feasible in undeveloped chicks. Capsaicin inhibited the activation of extracellular signal-regulated kinases (ERK) without significantly impacting p38 kinases. In young adult mice, capsaicin reduced the amount of newly produced cells in the dentate gyrus of the hippocampus but had no significant effect on learning and memory function. (Kong *et al*., 2010,). The observed effects on the brain imply that capsaicin may influence the neural development of chick embryos which aligns with the results observed by Kong 2010 *loc. cit.*

Caffeine

The embryos inoculated with caffeine all showed retarded growth. The effects were different from that of capsaicin. However, like capsaicin, the development of the surrounding blood vessels and consistency of the yolk was maintained.

1. **5%:** The embryos infected with 5% caffeine were the least damaged, demonstrating some brain and ocular development. Nonetheless, no more structures were clearly apparent. The head showed more development, resulting in a bigger size than the rest of the body and the body appeared to be somewhat translucent.
2. **10%:** The embryos inoculated with 10% Caffeine exhibited a more pronounced effect of the toxin as the size was significantly smaller compared to the control and the 5% Caffeine-inoculated embryos. While all the organs showed retarded growth, their differentiation was unaffected. The brain, eye, and other organs were visible but smaller in size and underdeveloped.

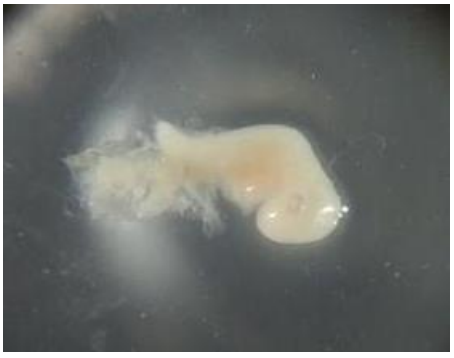

Image 1.7: Embryo inoculated with 10% Caffeine.

3. **100%:** Caffeine had a severe effect on embryos, causing incomplete differentiating and preventing the development of any organ. The eye, brain, and other organs were invisible. The embryo maintained a roughly similar shape to that of the control, but it was significantly smaller in size.

Caffeine's impact on neural development in chick embryos has been demonstrated, specifically by

selectively inhibiting the uplifting of neural folds, preventing the complete closure of the neural tube (Lee, *loc. cit*.). Furthermore, caffeine exhibits an effect on neurotransmitters and shows a marked inability to be metabolized in the embryonic brain (Li, *loc. cit*.). The observed outcomes, particularly at higher concentrations (10% and 100%), align with these findings by Gilani *et al*. in 1993, whereas samples with 5% caffeine inoculation did not exhibit similar effects. This supports the idea that caffeine, especially at higher concentrations, can induce significant morphological and developmental flaws in examined embryos.

Nicotine

Nicotine exposure had a significant and negative impact on embryo development. At both 10% and 100%, the eggs showed no indications of growth and semi-solid yolks. In the presence of 10% nicotine, embryo development was modest, and the yolk was slightly watery as observed in Figure 1.8. As shown in Figure 1.9 at 100% nicotine, there was no discernible growth, and the yolk had entirely solidified. The lack of visible embryos in these samples made it impossible to measure their development. In the case of the eggs inoculated with 5% Nicotine, both growth and development had come to a complete halt. However, the yolk remained in a liquid state, and some blood vessels were somewhat developed. Despite these observations, no embryo was visible.

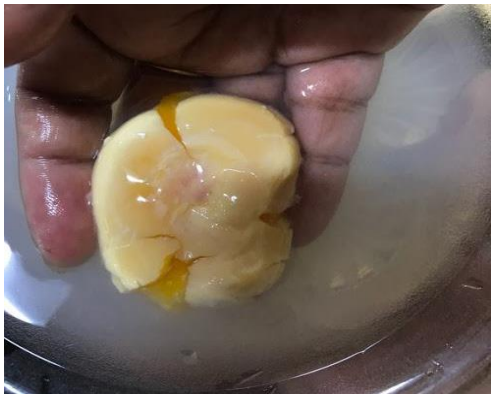

Figure 1.8: Embryo Inoculated with 10% Nicotine.

Figure 1.9: Embryo Inoculated with 100% Nicotine.

The embryos displayed high toxicity levels across all three nicotine doses, evident from the solidified yolk, suggesting that the addition of nicotine caused a certain degree of heat. A previous experiment by Rosenbruch, *loc. cit.* indicates that nicotine influences the growth of the liver and cardiovascular systems. Nicotine has a significant effect on embryo axial rotation. Atypical axial rotation was linked to partial closure of the embryonic neural tube in the cervical region, but not in other parts of the tube resulting in the brain development of the embryo. (England *et al*., 2017, Bohn *et al*., 2017)

CONCLUSION

Capsaicin seemed to affect neural and internal development concerning various organs. This determination was made based on the visibly reduced size and distinct red deposition within the chick embryo. Future research could delve into the uptake mechanism of capsaicin by a developing chick embryo, considering that the toxin used was a crude extract with potential impurities. Employing more efficient purification techniques in subsequent studies would be beneficial. Further investigations can aim to improve the understanding of these factors and refine experimental conditions for a more accurate assessment of capsaicin's effects on developing chick embryos.

The effects of caffeine showed a slight variation when compared to the previously conducted experiments (Li, *loc cit*.). Although the growth was significantly hampered, there was no visible neurological damage. Further research with a wider range of concentrations would be required. Caffeine has been shown in studies to reduce calcium uptake loss. Caffeine quickly became one of the most well-known risk factors for osteoporosis. (Heaney, 2002) and Caffeine has also been linked to bone damage through calcium metabolism disruption, altered vitamin D responses, and other pathways. In clinical and population-based investigations, the effect of coffee consumption on bone metabolism. (Berman *et al*., 2022) Additionally, histopathological analysis of individual organs at each stage can be carried out to study and observe the effects of the toxin on each organ.

The concentrations of nicotine utilized in the experiment were uniformly highly toxic to the embryos. To gain a deeper understanding of the heating effect lower nicotine concentrations, specifically below 5% nicotine have to be established. The inconclusive results from nicotine in the current study, where higher concentrations showed no growth at all and the 5% concentration showed only partial development of blood vessels, indicate the need for additional investigations at lower concentrations to establish more definitive conclusions.

This project lays the groundwork for potential future research into the toxic effects of Caffeine, capsaicin, and nicotine. The experiment can serve as a foundation for

exploring further the effects of capsaicin. It is worth noting that the capsaicin used in this study was a crude extract, containing impurities that might have had synergistic effects on its toxicity. Investigating these impurities could be a focus of future research. Additionally, future studies might explore the use of solvents other than water. Caffeine, though extensively studied in embryonic toxicology, yielded results in this experiment that deviated from previous research. (Li, *loc. cit*.,) This discrepancy could be explored further in subsequent research endeavors. Overall, this project serves as a starting point for furthering our understanding of the effects of these substances on embryonic development, potentially revealing new insights and applications that provide a foundation for toxicological effects in humans, as these toxins are commonly used in our daily lives.

ACKNOWLEDGMENTS

The authors would like to thank the Principal Dr. Krutika B. Desai for providing us with the guidance and the non-teaching staff department of Zoology SVKMs' Mithibai college for providing the laboratory where the work was completed of the project.

REFERENCES

1. Hiura, A., & Sakamoto, Y. Effect of capsaicin on neurites of cultured dorsal root ganglia and isolated neurons of chick embryos. Neuroscience Letters, 1987. https://doi.org/10.1016/0304-3940(87)90251-5
2. Ashwini, D., Sree, G. U., Ajitha, M. A., & Rao, V. U. M. Extraction of Capsaicin from Capsicum frutescens. L and its estimation by the RP-HPLC method. World Journal of Pharmacy and Pharmaceutical Sciences, 2015; 4(9): 839–848. https://www.cabdirect.org/cabdirect/abstract/20153315998
3. Barone, J., & Roberts, H. Caffeine consumption. *Food and Chemical Toxicology*, 1996; *34*(1): 119–129. https://doi.org/10.1016/0278-6915(95)00093-3
4. Bohn, N., Humphrey, V., & Omelchenko-Comer, N. Effects of nicotine on chicken embryonic development. Proceedings of the West Virginia Academy of Science, 2017; 89(2). https://doi.org/10.55632/pwvas.v89i2.149
5. Brandon, T. H., Goniewicz, M. L., Hanna, N. H., Hatsukami, D. K., Herbst, R. S., Hobin, J. A., Ostroff, J. S., Shields, P. G., Toll, B. A., Tyne, C., Viswanath, K., & Warren, G. W. Electronic Nicotine Delivery Systems: A Policy Statement From the American Association for Cancer Research and the American Society of Clinical Oncology. *Journal of Clinical Oncology*, 2015; *33*(8): 952–963. https://doi.org/10.1200/jco.2014.59.4465
6. Berman, N., Honig, S., Cronstein, B. N., & Pillinger, M. H. The effects of caffeine on bone mineral density and fracture risk. Osteoporosis International, 2022; 33(6): 1235–1241. https://doi.org/10.1007/s00198-021-05972-w
7. Cardinale, A., Nastrucci, C., Cesario, A., & Russo, P. Nicotine: specific role in angiogenesis, proliferation and apoptosis. *Critical Reviews in Toxicology*, 2011b; *42*(1): 68–89. https://doi.org/10.3109/10408444.2011.623150
8. *Caffeine*. (n.d.). CAMH. https://www.camh.ca/en/health-info/mental-illness-and-addiction-index/caffeine#:~:text=Caffeine%20is%20the%20most%20widely,to%20238%20mg%20per%20day. [Accessed on 18 Jan 2024]
9. England, L. J., Bunnell, R., Pechacek, T. F., Tong, V. T., & McAfee, T. Nicotine and the Developing Human. *American Journal of Preventive Medicine*, 2015; *49*(2): 286–293. https://doi.org/10.1016/j.amepre.2015.01.015
10. Fakhr El-Din M. Lashein and Amin Abdou Seleem., Effect of Caffeine on the developing chick embryos: Morphology and Skeletal Alterations. *Egypt. J. Zool*, 2012; 58: 105-124.
11. Galli, C., Spano, P., & Szyszka, K. Accumulation of caffeine and its metabolites in rat fetal brain and liver. *Pharmacological Research Communications*, 1975; *7*(3): 217–221. https://doi.org/10.1016/0031-6989(75)90021-1
12. Gilani, S., Giovinazzo, J., & Persaud, T. Embryopathic effects of caffeine in the chick. *Experimental Pathology*, 1983. https://doi.org/10.1016/s0232-1513(83)80044-9
13. Gilbert, S. F. *Developmental Biology*, 1988; 6.
14. Gonçalves, E. M., & Da Piedade, M. E. M. Solubility of nicotinic acid in water, ethanol, acetone, diethyl ether, acetonitrile, and dimethyl sulfoxide. *The Journal of Chemical Thermodynamics*, 2012; *47*: 362–371. https://doi.org/10.1016/j.jct.2011.11.023
15. Haass, M., & Kübler, W. Nicotine and sympathetic neurotransmission. *Cardiovascular Drugs and Therapy*, 1997; *10*(6): 657–665. https://doi.org/10.1007/bf00053022
16. *The Health Consequences of Smoking - 50 Years of Progress: A Report of the Surgeon General*, 2014.
17. Heaney, R. P. Effects of caffeine on bone and the calcium economy. Food and Chemical Toxicology, 2002; 40(9): 1263–1270. https://doi.org/10.1016/s0278-6915(02)00094-7
18. Hicks, M. B., Hsieh, Y. P., & Bell, L. N. Tea preparation and its influence on methylxanthine concentration. *Food Research International*, 1996b; *29*(3–4), 325–330. https://doi.org/10.1016/0963-9969(96)00038-5
19. Johnson, W. Final report on the safety assessment of Capsicum annuum extract, Capsicum annuum fruit extract, Capsicum annuum resin, Capsicum annuum fruit powder, Capsicum Frutescens fruit, Capsicum frutescens fruit extract, Capsicum frutescens resin, and Capsaicin. *International Journal of Toxicology*, 2007. https://worldveg.tind.io/record/21728

20. Kimmel, C. A., Kimmel, G. L., White, C., Grafton, T. F., Young, J. F., & Nelson, C. J. Blood flow changes and conceptual development in pregnant rats in response to caffeine. *Fundamental and Applied Toxicology*, 1984; *4*(2): 240–247. https://doi.org/10.1016/0272-0590(84)90125-8
21. Kong, K. H., Kim, H. K., Song, K. S., Woo, Y., Choi, W. S., Park, H. R., Park, M., Kim, M. E., Kim, M., Ryu, J. S., Kim, H. S., & Lee, J. Y. Capsaicin impairs proliferation of neural progenitor cells and hippocampal neurogenesis in young mice. Journal of Toxicology and Environmental Health, Part A: Current Issues, 2010; 73(21–22): 1490–1501. https://doi.org/10.1080/15287394.2010.511572
22. Korn, M. J., & Cramer, K. S. Windowing Chicken Eggs for Developmental Studies. *Journal of Visualized Experiments*, 2007; *8*. https://doi.org/10.3791/306
23. Lagrue, G., Lebargy, F., & Cormier, A. Des récepteurs nicotiniques à la dépendance tabagique : Perspectives thérapeutiques. *Colloque D'alcoologie Lipha-Santé*, 2001; *23*(2).
24. Lee, H., Nagele, R. G., & Pietrolungo, J. F. Toxic and teratologic effects of caffeine on explanted early chick embryos. *Teratology*, 1982. https://doi.org/10.1002/tera.1420250104
25. Li, X., He, R., Yang, Q., Tsoi, B., Li, Y., Ma, Z., Yang, X., & Kojima, H. Caffeine interferes embryonic development through over-stimulating serotonergic system in chicken embryo. *Food and Chemical Toxicology*, 2012; *50*(6): 1848–1853. https://doi.org/10.1016/j.fct.2012.03.037
26. Ma, Z., Yang, Q., Wang, G., Li, X., He, R., Chuai, M., Kojima, H., & Yang, X. Exploring the Caffeine-Induced teratogenicity on neurodevelopment using early chick embryo. *PLOS ONE*, 2012; *7*(3): e34278. https://doi.org/10.1371/journal.pone.0034278
27. Nathanson, J. A. Caffeine and Related Methylxanthines: Possible Naturally Occurring Pesticides. *Science*, 1984; *226*(4671): 184–187. https://doi.org/10.1126/science.6207592
28. Nehlig, A., Daval, J., & Debry, G. Caffeine and the central nervous system: mechanisms of action, biochemical, metabolic and psychostimulant effects. *Brain Research Reviews*, 1992; *17*(2): 139–170. https://doi.org/10.1016/0165-0173(92)90012-b
29. "Nicotine (PIM)". Inchem.org. Retrieved, 2012; 12: 19.
30. *Nicotine - Alcohol and Drug foundation*. (n.d.), 2024. https://adf.org.au/drug-facts/nicotine/
31. Okamoto, M., Kita, T., Okuda, H., Tanaka, T., & Nakashima, T. Effects of Aging on Acute Toxicity of Nicotine in Rats. *Pharmacology & Toxicology*, 1994; *75*(1): 1–6. https://doi.org/10.1111/j.1600-0773.1994.tb00316.x
32. O'Neill, J., Brock, C., Olesen, A. E., Andresen, T., Nilsson, M., & Dickenson, A. H. Unravelling the Mystery of Capsaicin: A Tool to Understand and Treat Pain. *Pharmacological Reviews*, 2012; *64*(4): 939–971. https://doi.org/10.1124/pr.112.006163
33. Pavia, D. L., Lampman, G. M., Kriz, G. S., & Engel, R. G. *Introduction to Organic Laboratory Techniques: A Microscale Approach*, 1999.
34. Rodu, B. The scientific foundation for tobacco harm reduction, 2006-2011. *Harm Reduction Journal*, 2011; *8*(1): 19. https://doi.org/10.1186/1477-7517-8-19
35. Rosenbruch, M., Kniepen, J., & Weishaupt, C. The early chick embryo as a model to evaluate cardiovascular effects of adrenaline and nicotine. *Toxicology in Vitro*, 1993. https://doi.org/10.1016/0887-2333(93)90062-a
36. Schaber, P., Larkin, J. E., Pines, H. A., Berchou, K., Wierchowski, E. E., Marconi, A., & Suriani, A. Supercritical Fluid Extraction versus Traditional Solvent Extraction of Caffeine from Tea Leaves: A Laboratory-Based Case Study for an Organic Chemistry Course. *Journal of Chemical Education*, 2012; *89*(10): 1327–1330. https://doi.org/10.1021/ed200536e
37. Schmidt, R. J., Romitti, P. A., Burns, T. L., Browne, M. L., Druschel, C. M., & Olney, R. S. Maternal caffeine consumption and risk of neural tube defects. Birth Defects Research, 2009; 85(11): 879–889. https://doi.org/10.1002/bdra.20624
38. Schmidt, R. J., Romitti, P. A., Burns, T. L., Murray, J. C., Browne, M. L., Druschel, C. M., & Olney, R. S. Caffeine, selected metabolic gene variants, and risk for neural tube defects. Birth Defects Research, 2010; 88(7): 560–569. https://doi.org/10.1002/bdra.20681
39. Schraufnagel, D. E., Blasi, F., Drummond, M., Lam, D., Latif, E., Rosen, M. A., Sansores, R., & Van Zyl-Smit, R. N. Electronic Cigarettes. A Position Statement of the Forum of International Respiratory Societies. *American Journal of Respiratory and Critical Care Medicine*, 2014; *190*(6): 611–618. https://doi.org/10.1164/rccm.201407-1198pp
40. Suter, M., & Aagaard, K. M. The impact of tobacco chemicals and nicotine on placental development. *Prenatal Diagnosis*, 2020; *40*(9): 1193–1200. https://doi.org/10.1002/pd.5660
41. Sharma, S. K., Vij, A. S., & Sharma, M. Mechanisms and clinical uses of capsaicin. European Journal of Pharmacology, 2013; 720(1–3): 55–62. https://doi.org/10.1016/j.ejphar.2013.10.053
42. Tanaka, H., Nakazawa, K., & Arima, M. Effects of maternal caffeine ingestion on the perinatal cerebrum. *Neonatology*, 1987; *51*(6): 332–339. https://doi.org/10.1159/000242672
43. Tumimbang, G. V., Espiritu, W. A., Silong, R. Extraction of Caffeine from Coffee. Chemistry 35.1, University of the Philippines Manila, 2014.
44. Varlet, V., Farsalinos, K., Augsburger, M., Thomas, A., & Etter, J. Toxicity Assessment of Refill Liquids for Electronic Cigarettes. *International Journal of Environmental Research and Public Health*, 2015;

12(5): 4796–4815. https://doi.org/10.3390/ijerph120504796
45. What Made Chili Peppers So Spicy? Talk of the Nation, 2008; 15.
46. Wilde r, Natalie; Daley, Claire; Sugarman, Jane; Partridge, James. "Nicotine without smoke: Tobacco harm reduction". UK: Royal College of Physicians, 2016; 125.
47. World Health Organization, 2017. Tobacco. [online] Available at: http://www.who.int/mediacentre/factsheets/fs339/en/ [Accessed 23 Jul. 2017].
48. Li, X., He, R., Qin, Y., Tsoi, B., Li, Y., Ma, Z., Yang, X., & Kurihara, H. Caffeine interferes embryonic development through over-stimulating serotonergic system in chicken embryo. *Food and Chemical Toxicology*, 2012. https://doi.org/10.1016/j.fct.2012.03.037