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RESEARCH ARTICLE

Effects of *In-Ovo* Chrysin Injection to Quail Eggs on Hatchability, Production Parameters, and Immunity

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Abstract

This study investigated the effects of in ovo Chrysin injection on hatchability, embryonic mortality, body weight, feed conversion ratio (FCR), and immune responses in quails. A total of 720 fertilized eggs were randomly assigned to four groups: Control (0.1 mL distilled water) and Chrysin-treated groups (0.1 mL containing 0.25 mg, 0.50 mg, or 0.75 mg Chrysin per egg). Hatchability and hatching efficiency were unaffected by Chrysin treatment, although late embryonic mortality was significantly higher in the 0.75 mg group. Post-hatch evaluations showed significantly greater body weights on days 14 and 42 in the 0.25 mg and 0.50 mg groups compared to the control. These groups also exhibited improved FCR values, while feed intake remained unchanged across groups. Immunological analysis revealed a significant increase in IgA levels in the 0.75 mg group, with no significant effects on IgM or IgG levels. Overall, *in ovo* Chrysin supplementation at 0.25 mg and 0.50 mg enhanced growth performance and feed efficiency without adversely affecting hatchability, while the 0.75 mg increased embryonic mortality, indicating a need for dose optimization. These findings suggest that Chrysin holds potential as a biotechnological tool in poultry production, though further studies are required to refine dosing strategies and investigate its long-term impacts on performance and immunity.

Keywords: *Chrysin, Feed conversion ratio, Hatchability, Immunity, In-ovo*

Introduction

Avian embryonic development differs fundamentally from that of mammals, as it relies entirely on the nutrients stored within the egg, with no additional natural sources available to meet the embryo's nutritional needs [1]. Although the egg is nutritionally complete and capable of supporting the embryo throughout the incubation period [1,2], recent intensive selection in commercial poultry has resulted in increased metabolic rates during embryogenesis, thereby elevating the nutritional demands of the developing embryo. Insufficient nutrition during this critical period can impair embryonic development, reduce hatchability, and adversely affect post-hatch growth [3]. Moreover, the limited nutrient supply during incubation can constrain the growth potential of newly hatched chicks, preventing them from reaching their optimal developmental capacity [1,4].

To address these challenges, a number of studies have explored strategies to supplement the limited nutritional reserves available to embryos [1,2,5]. One promising approach is *in ovo* feeding, which involves the injection of liquid nutrients into specific areas of the egg, such as the albumen, amniotic fluid, yolk sac, or air sac, at various stages of incubation [4,6-8]. *In ovo* feeding has emerged as a viable strategy to enhance embryonic nutrition, potentially mitigating perinatal nutritional deficiencies and improving hatchability [9,10].

Among the various substances explored for *in ovo* feeding, plant-derived flavonoid compounds have garnered particular attention due to their pharmacological effects, antioxidant activities, and enzyme inhibition properties [11]. Chrysin *(5,7-dihydroxyflavone)*, a flavonoid found in plants such as *Passiflora caerulea, Passiflora incarnata*, and *Oroxylum indicum*, is noted for its wide range of biological effects, including antioxidant, anti-inflammatory, anti-cancer, anti-aging, anti-allergic, anti-diabetic, and neuro-protective properties [11-15]. Chrysin has also been shown to exhibit potent antioxidant activity, and numerous studies have investigated its effects of adding Chrysin to the diets of animals on animal performance and its protective

effects against various toxic agents [16-19]. However, research on the effects of *in ovo* Chrysin administration remains limited. A study by Khaligh et al.[20] indicated that *in ovo* administration of Chrysin did not significantly impact hatchability or the post-hatch performance of broiler chicks from 0 to 11 days of age. Conversely, Kurt et al.[21] observed a significant reduction in cataract formation in Chrysin-treated embryos, suggesting that its antioxidant and anti-apoptotic properties may contribute to this beneficial effect.

This study aims to investigate the effects of *in ovo* Chrysin administration on immune function and performance in quails up to 42 days of age. Specifically, it evaluates the impact of Chrysin injection into fertilized quail eggs on hatchability, embryonic mortality, production parameters such as body weight and feed conversion ratio (FCR), as well as immune responses. This study seeks to provide further insight into the potential of Chrysin as an *in ovo* nutrient supplement that could enhance both embryonic development and post-hatch health.

Material and Methods

Ethical Statement

The research was conducted at the Atatürk University Food and Livestock Application and Research Center Poultry Unit and was ethically approved by the Atatürk University Local Ethics Council of Animal Experiments (29.03.2022, 2022/4, Decision number: 64).

Experimental Procedures

A total of 750 eggs were collected from Japanese quail breeders *(Coturnix coturnix japonica)* aged 16 to 20 weeks, comprising 60 males and 180 females, over a period of 7 days. Prior to incubation, 30 eggs exhibiting broken or cracked shells and those not meeting ideal shape and size criteria were discarded. The experiment was designed with four groups (one control and three differing doses of Chrysin). A total of 720 eggs were randomly allocated into four groups of 180 eggs each. These eggs were placed in a single-stage incubator during the improvement stage (0-15 days), where the temperature was maintained at 37.5±0.3°C and relative humidity at 65±5%. The eggs were turned 90 degrees forward and backward at a 45-degree angle from the vertical axis every hour.

Before transitioning to the hatching period (the last 3 days of incubation), the following *in-ovo* injection treatments were administered:

Group 1 (Control): 0.1 mL of distilled water

Group 2: 0.1 mL injection containing 0.25 mg Chrysin per egg

Group 3: 0.1 mL injection containing 0.50 mg Chrysin per egg

Group 4: 0.1 mL injection containing 0.75 mg Chrysin per egg

Chrysin doses were dissolved in a physiological saline solution to a final volume of 1 mL. Eggs were disinfected with 70% ethanol and pierced at the flatter end (air cell) before manual injection using a 26 G syringe to a depth of approximately 5 mm. After injection, the holes were sealed with nail polish and disinfected again with 70% ethanol [7,21]. The injected eggs were then placed back into the single-stage incubator.

During the final 3 days of incubation, the temperature was adjusted to 36.5±0.3°C, with relative humidity set at 75±5%. Upon hatching, chicks were individually weighed to calculate average body weight. Ten chicks per treatment group were euthanized using mild sevoflurane anesthesia, and liver samples were collected and stored at -20°C for subsequent biochemical analyses.

Hatchability Parameters

Hatchability was calculated as the proportion of successfully hatched chicks relative to the total number of eggs incubated. Fertilization rate was determined as the proportion of fertile eggs among the total eggs incubated. Hatching success, also referred to as hatching efficiency, was defined as the proportion of chicks hatched from the total number of fertile eggs.

Non-hatched eggs were broken to assess the presence of embryos, and embryonic mortality was classified according to the stage of incubation as early (1-6 days), intermediate (7-14 days), or late (15-18 days).

Raising Chicks

Healthy chicks were reared in separate brooder cages according to their respective treatment groups for the initial two weeks. To ensure post-hatch uniformity, a total of 360 chicks -90 from each experimental group- were placed in cages. During this period, a starter diet containing 23% crude protein (CP) and 3.000 kcal/kg metabolizable energy (ME) was provided. After the second week, the chicks were transferred to multi-tier rearing cages, with mixed gender assigned according to their treatment groups. The housing system consisted of four cage units, each comprising three tiers, with five cage cells per tier. Six chicks were housed in each cage cell. The grower diet was designed to meet the nutritional needs of quails, containing 20% CP and 3.250 kcal/kg ME. Feed and water were provided ad libitum throughout the study. Environmental conditions were controlled: the ambient temperature was maintained at 32-33°C for the first three days, then gradually decreased by 1-2°C per week until stabilized at 24°C. A lighting regimen of 23 h of light and 1 h of darkness per day was implemented for the duration of the experiment. Daily feed intake was monitored, and body weight along with weight gain were recorded

at 10:00 AM on the first day of each week. Feed intake and FCR were calculated. The study concluded when the quails reached six weeks of age, at which point the age at sexual maturity, defined as the age of first egg-laying, was documented.

Liver Biochemical Analyses

Standards and samples of immunoglobulin A (IgA), immunoglobulin G (IgG), and immunoglobulin M (IgM) were pipetted into wells of a microplate pre-coated with specific antibodies. The IgA, IgG, and IgM present in the samples bound to the immobilized antibodies. Enzyme-linked secondary antibodies were then added to bind with the primary antibodies, followed by the addition of an enzyme substrate, resulting in a color change proportional to the concentrations of IgA, IgG, and IgM. The color intensity was measured at 450 nm [22].

The calculation was performed using a regression equation derived from a standard curve, with absorbance values plotted on the X-axis and corresponding standard concentrations on the Y-axis. The experiment, including reagent preparation and calculation procedures, was conducted using consistent protocols, the same brand of reagents, and identical measurement steps throughout.

Statistical Analysis

Data were analyzed using SPSS 12.0. The chi-square test was used for embryonic mortality and hatchability analysis; One-Way ANOVA for body weight changes, feed conversion ratios, and average feed intake; and General Linear Model for biochemical data. Results with $P<0.05$ were considered significant.

Results

The research findings indicated that the 0.75 mg Chrysin treatment group exhibited the lowest hatchability and hatching efficiency rates; however, these differences were not statistically significant ($P>0.05$). The hatchability rates for the control, 0.25 mg, 0.50 mg, and 0.75 mg Chrysin treatments were 72.78%, 72.22%, 74.44%, and 62.78%, respectively. Similarly, the hatching efficiency rates were 86.75%, 83.87%, 84.81%, and 75.84%, respectively *(Table 1)*.

As the experimental treatment was applied on the 15th day of incubation, it had no effect on fertility rates or early- to mid-term embryonic mortality. Regarding embryonic mortality, the highest incidence was observed during the late embryonic period. Late embryonic death rates for the experimental groups were 8.61%, 11.61%, 10.76%, and 18.79%, respectively. The rate of late embryonic death was significantly higher in the 0.75 mg treatment group compared to the other groups ($P<0.05$) *(Table 1)*.

A statistically significant difference in body weight was observed between treatment groups on days 14 and 42 ($P<0.05$). On day 14, quails in the 0.25 mg Chrysin group exhibited the lowest body weight but demonstrated the highest weight gain throughout the experiment. By day 42, quails in all Chrysin treatment groups (0.25 mg, 0.50

Table 1. *Hatchability performance and embryo mortality rates (%) as a function of treatment*

Parameter		Control	0.25 mg	0.50 mg	0.75 mg	P
Hatchability Performance	Hatchability (%)	72.78	72.22	74.44	62.78	0.065
	Fertilization rate (%)	83.89	86.11	87.78	82.78	0.544
	Hatching efficiency (%)	86.75	83.87	84.81	75.84	0.062
Embryo mortality rate (%)	Early (1 to 6 d)	1.32	2.58	1.90	2.68	0.755
	Intermediate (7 to 14 d)	3.31	1.94	2.53	2.68	0.908
	Late (15 to 18 d)	8.61^{b}	11.61^{b}	10.76^{b}	18.79^{a}	0.044

$^{a, b}$*Different letters within one row are significantly different (P<0.05)*

Table 2. *Average weekly body weights and standard errors of the trial groups ($\bar{x} \pm SE$)*

Day	Body Weight (g)				
	Control	0.25 mg	0.50 mg	0.75 mg	P
14	55.43±1.28^{ab}	52.51±1.85^{b}	56.97±1.46^{ab}	59.69±1.31^{a}	0.015
21	97.39±1.53	95.39±1.08	98.65±2.61	99.89±1.89	0.324
28	136.39±1.94	139.18±1.80	141.22±3.71	141.88±2.67	0.407
35	170.58±2.09	178.88±2.39	180.42±4.17	176.49±1.88	0.055
42	202.62±1.22^{b}	214.87±2.70^{a}	215.15±3.69^{a}	208.58±1.80^{ab}	0.001

$^{a, b}$ *Different letters within one row are significantly different (P<0.05)*

Table 3. *Average weekly daily feed intake and standard errors of the trial groups ($\bar{x}$± SE)*

Day	Daily Feed Intake (g/quail)				
	Control	0.25 mg	0.50 mg	0.75 mg	P
14-21	20.24±1.16	21.67±1.19	23.46±1.94	22.74±1.46	0.411
21-28	23.70±1.24	27.10±1.06	25.78±1.03	25.53±1.01	0.165
28-35	35.55±1.09	33.14±1.30	32.05±0.96	34.53±1.14	0.165
35-42	36.43±0.81	35.49±1.40	32.04±1.18	36.47±0.50	0.734
14-42	28.98±0.54	29.35±0.47	29.08±0.87	29.82±0.53	0.778

Table 4. *Average weekly feed conversion ratio and standard errors of the trial groups ($\bar{x}$± SE)*

Day	Feed Conversion Ratio				
	Control	0.25 mg	0.50 mg	0.75 mg	P
14-21	3.45±0.22	3.57±0.19	3.95±0.30	3.99±0.28	0.320
21-28	4.39±0.27	4.47±0.29	4.32±0.19	4.53±0.40	0.968
28-35	7.34±0.29[a]	5.92±0.29[b]	5.98±0.37[b]	7.22±0.46[a]	0.004
35-42	8.32±0.47	7.12±0.30	7.38±0.41	8.00±0.21	0.085
14-42	5.51±0.56[a]	5.08±0.97[b]	5.15±0.10[b]	5.61±0.85[a]	<0.001

[a, b] *Different letters within one row are significantly different (P<0.05)*

Table 5. *Average IgA, IgM and IgG levels and standart errors of the trial groups ($\bar{x}$± SE)*

Type	Immunoglobulin Levels (mg/ml)				
	Control	0.25 mg	0.50 mg	0.75 mg	P
IgA	62.30±1.20[b]	63.10±1.18[b]	61.64±1.18[b]	69.55±1.18[a]	<0.001
IgM	483.61±16.61	483.32±16.38	447.79±16.38	504.36±16.38	0.118
IgG	377.79±12.25	364.37±12.08	357.42±12.58	394.84±12.08	0.148

[a, b] *Different letters within one row are significantly different (P<0.05)*

mg, and 0.75 mg) had higher body weights compared to the control group. Specifically, the final body weights at the end of the experiment were 202.62 g for the control group, 214.87 g for the 0.25 mg group, 215.15 g for the 0.50 mg group, and 208.58 g for the 0.75 mg group. The highest body weights were recorded in the 0.50 mg and 0.25 mg Chrysin treatment groups, with a significant difference (P<0.01) observed between these groups and the control *(Table 2)*.

The daily feed intake of quails across treatment groups is presented in *Table 3*. Feed intake exhibited an increasing trend over time in all groups; however, the differences among the groups were not statistically significant (P>0.05). During the experimental period (14-42 days), the average daily feed intake was recorded as 28.98 g, 29.35 g, 29.08 g, and 29.82 g for the control group and the groups receiving 0.25 mg, 0.50 mg, and 0.75 mg of Chrysin, respectively.

FCR are presented in *Table 4*. No significant differences in FCR were observed among the groups during individual weeks, except for the 3rd week (P>0.05). However, statistically significant differences were found in FCR values over the entire experimental period (14-42 days) (P<0.001). The average FCR values for the control group and the groups receiving 0.25 mg, 0.50 mg, and 0.75 mg of Chrysin were 5.51, 5.08, 5.15, and 5.61, respectively. The 0.25 mg and 0.50 mg Chrysin groups demonstrated better FCR values compared to the control and 0.75 mg Chrysin groups. Additionally, FCR values increased over time in all groups.

The IgA levels in liver tissues of chicks were measured as 62.30±1.20 mg/ml, 63.10±1.18 mg/mL, 61.64±1.18 mg/mL, and 69.55±1.18 mg/mL for the control group and the groups receiving 0.25 mg, 0.50 mg, and 0.75 mg of Chrysin, respectively. Statistically significant differences in IgA levels were observed among the groups (P<0.001). However, no significant differences were found in IgM and IgG levels *(Table 5)*.

The age at sexual maturity, defined as the age at first egg, was recorded as 40, 41, 39, and 37 days for the control group and the 0.25 mg, 0.50 mg, and 0.75 mg Chrysin groups, respectively.

Discussion

This study examined the effects of *in ovo* Chrysin injection at three different doses. As the injections occurred on the 15th day of the embryonic period, they had no impact on fertilization rates, early- and mid-term embryonic mortality.

The results revealed no statistically significant relationship between *in-ovo* Chrysin injections and hatchability or hatching efficiency. Although a trend towards lower hatchability was observed, the lack of statistical significance suggests that the impact of Chrysin on hatching success is relatively mild within the tested dose ranges. Notably, the 0.75 mg dose was associated with lower hatchability rates and increased late-stage embryonic mortality (18.79%), indicating potential developmental challenges at higher doses. In contrast, previous studies have reported improved hatching outcomes with other substances. For instance, Genc et al.[7] found that a 0.25 mg Rutin injection yielded the highest hatching performance in quail eggs, while Ghane et al.[23] demonstrated that in-ovo feeding with vitamins C and E significantly improved hatchability in broiler eggs. Similarly, Taha et al.[24] reported that a 0.5 mL *in-ovo* royal jelly injection on the 7th day of incubation enhanced hatching performance.

Late embryonic deaths observed in this study may be attributed to the timing and method of *in-ovo* application rather than the antioxidant properties of Chrysin itself. Since the injections were performed after the first 15 days of incubation, they could have disrupted critical stages of embryonic development. This is consistent with findings from Subramaniyan et al.[25], who reported that *in-ovo* L-arginine injection on the 14th day of incubation in chicken eggs positively influenced survival and hatching rates, whereas applications closer to hatching showed diminished benefits.

The increased late embryonic mortality in the 0.75 mg Chrysin group (18.79%) compared to the control (8.61%), 0.25 mg (11.61%), and 0.50 mg (10.76%) groups highlights a dose-dependent effect. While Chrysin's antioxidant and anti-inflammatory properties may offer potential benefits, higher concentrations could negatively affect embryonic development during the later stages of incubation. These adverse effects may stem from overdose of Chrysin, which can disrupt the balance of oxidative stress, alter cellular metabolism, and affect gene expression. Excessive doses might impair tissue and organ formation, leading to higher mortality in the final stages of embryogenesis [26,27].

The weekly body weight measurements demonstrate the potential of Chrysin as an *in ovo* dietary supplement to enhance the growth performance of quails. The results indicate that *in ovo* administration of Chrysin significantly improves growth performance, particularly in the groups receiving 0.25 mg and 0.50 mg doses. At the end of the trial (42 days), the highest body weights were observed in these two groups, whereas the control group exhibited the lowest final body weight. These findings are consistent with previous studies highlighting the beneficial effects of flavonoids on growth performance through their antioxidant, anti-inflammatory, and metabolic regulatory roles [25,28]. Coskun et al.[28] reported that *in ovo* DL-methionine injection increased chick weight by 3.8%. Considering the positive correlation between chick weights and subsequent body weights [29], it is likely that *in ovo* injection of Chrysin can similarly enhance adult body weights. Furthermore, Hassan et al.[30] observed that *in ovo* injection of different Cu sources improved broiler chicken body weights, while Dang et al.[31] reported improved development in goose embryos with disaccharide and methionine injections. Similarly, Abdel-Halim et al.[32] demonstrated that *in ovo* injection of folic acid and glucose (0.2 mg folic acid + 125 mg glucose) enhanced carcass characteristics by increasing growth rates. The observed improvements in body weight in the Chrysin-treated groups can be attributed to the compound's ability to reduce oxidative stress during critical periods of embryonic development and early growth [27]. By scavenging free radicals and supporting cellular functions, Chrysin likely enhances energy efficiency and nutrient utilization, resulting in improved growth rates [27]. Reduced oxidative stress may promote better cell proliferation and organ development, potentially explaining the superior weight gain observed in the Chrysin-treated groups compared to the control. A noteworthy aspect of this study is the dose-dependent response. While the 0.25 mg and 0.50 mg doses of Chrysin optimized growth, the 0.75 mg dose resulted in slightly lower final body weights compared to the other Chrysin-treated groups. This suggests that the 0.75 mg dose may exceed the optimal range, potentially causing suboptimal effects due to metabolic imbalances or mild toxicity. This phenomenon is consistent with the hormesis theory, which proposes that moderate doses of bioactive compounds elicit beneficial effects, whereas higher doses may inhibit growth or induce stress [33].

Regarding feed intake no significant differences found between the groups, suggesting that Chrysin does not influence appetite or feed intake behavior in quails. However, a significant difference was observed in FCR maintenance during the experimental period (14-42 days). The lack of variation in feed intake, coupled with differences in FCR, may underscore Chrysin's potential role in enhancing nutrient utilization efficiency. The improved FCR values in the Chrysin-treated groups can likely be attributed to the compound's antioxidant properties, which may reduce oxidative stress during critical

developmental stages [34]. By improving cellular energy efficiency and nutrient absorption, Chrysin supports better growth performance without altering feed intake. Consistent with previous research, the positive effects observed in this study support the role of flavonoids and *in ovo* administration of bioactive compounds in enhancing metabolic health, feed intake, and growth performance in poultry [35-38]. The dose-dependent effects observed in this study are particularly noteworthy. The optimal FCR values in the 0.25 mg and 0.50 mg Chrysin groups suggest that these doses enhance nutrient metabolism and energy utilization. In contrast, the slightly higher FCR observed in the 0.75 mg group may indicate potential metabolic disruptions or mild toxicity at this dose [26,27]. This finding highlights the importance of determining an optimal dosage range for Chrysin supplementation to maximize its efficacy while avoiding adverse effects.

Immunity, or the immune response, serves as the defense mechanism against microorganisms such as viruses, bacteria, fungi, and parasites in all vertebrates, including poultry. It is crucial for preventing disease development and begins to develop during incubation. Chicks receive maternal antibodies from the yolk sac, which provide protection against microorganisms during early life [39]. The development of immunity during the chick period is vital, as it significantly impacts their survival and later performance [40,41]. Williams and Hopkins [42] highlighted that one advantage of *in-ovo* injection is the earlier stimulation of the immune system. Our study observed a significant difference in IgA levels, a key component of mucosal immunity, with the highest levels in the 0.75 mg Chrysin group. IgA plays a critical role in preventing bacteria and viruses from adhering to epithelial surfaces, a process known as immune exclusion [43]. Chrysin's antioxidant and anti-inflammatory properties may enhance immune responses, specifically mucosal immunity, by promoting higher antibody production [44]. The significant increase in IgA levels in the 0.75 mg Chrysin group suggests that this higher dose could improve mucosal immune function, possibly by modulating cytokine production or enhancing gut barrier integrity [43]. No significant differences were found between groups for IgM and IgG concentrations. The absence of differences in IgM and IgG levels across treatment groups suggests that Chrysin's effects on the immune system may be specific to mucosal immunity rather than systemic humoral immunity. This specificity could be attributed to Chrysin's potential influence on gut-associated lymphoid tissue (GALT), which primarily regulates IgA production [45] . Previous studies on *in ovo* injections to enhance chick immunity have yielded positive results. Gore and Qureshi [46] found that vitamin E injection into the amniotic fluid of 18-day-old embryos enhanced both cellular and humoral immunity. Tufarelli et al.[47] reported that *in ovo* injection of folic acid increased IgM and IgG levels, thereby strengthening the immune system. Goel et al.[8] observed positive effects on immunity with thiamine and pyridoxine injections, while Hassan et al.[30] indicated that *in ovo* injection of various Cu sources did not negatively affect immune parameters. Similarly, Subramaniyan et al.[25] concluded that *in ovo* L-arginine application on day 14 of incubation effectively stimulated the immune response by increasing IgM levels.

In conclusion, this study demonstrates that *in ovo* Chrysin supplementation at optimal doses (0.25 mg and 0.50 mg) enhances body weight, feed efficiency, and mucosal immunity in quails without adversely affecting hatchability. However, the findings also reveal dose-dependent effects, with higher doses (0.75 mg) increasing late embryonic mortality and diminishing growth performance, underscoring the importance of precise dose optimization. While Chrysin showed promising antioxidant and anti-inflammatory benefits, particularly in improving nutrient metabolism and immune defenses, its limited impact on systemic immunity and hatching success highlights the need for further research to refine dosing strategies and *in ovo* injection protocols.

Declarations

Availability of Data and Materials: Materials and data sets from the study are available upon request from the corresponding author (M. Genç).

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Ethical Statement: The research ethically approved by the Atatürk University Local Ethics Council of Animal Experiments (29.03.2022, 2022/4, Decision number: 64).

Competing Interests: The authors declare that they have no conflict of interest.

Declaration of Generative Artificial Intelligence (AI): The authors declare that the article, tables and figures were not written/created by AI and AI-assisted technologies.

Author Contributions: The conception and design of the study were conducted by MG. MG and UO were responsible for data acquisition, analysis, and interpretation, as well as drafting the manuscript. Biochemical analyses were performed by SK. The critical review and revision of the manuscript were collaboratively undertaken by MG, UO, and SK.

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