Szkoła Główna Gospodarstwa Wiejskiego
w Warszawie

Instytut Nauk o Zwierzętach

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Wpływ nanocząstek wybranych związków wapnia podawanych *in ovo* na rozwój zarodka kury oraz wyniki produkcyjne i jakość kości kurcząt brojlerów

Effect of selected calcium compounds nanoparticles administered *in ovo* on the chicken embryo development and production results and bone quality of broiler chickens

Praca doktorska

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Oświadczenie promotora pracy

Oświadczam, że niniejsza praca została przygotowana pod moim kierunkiem i stwierdzam, że spełnia ona warunki do przedstawienia jej w postępowaniu o nadanie stopnia naukowego.

Data 08. 03. 2022 r. Podpis promotora pracy ……

Oświadczenie autora pracy

Świadom odpowiedzialności prawnej oświadczam, że niniejsza praca doktorska została napisana przeze mnie samodzielnie i nie zawiera treści uzyskanych w sposób niezgodny z obowiązującymi przepisami.

Oświadczam również, że przedstawiona praca nie była wcześniej przedmiotem procedur związanych z uzyskaniem stopnia naukowego w wyższej uczelni.

Oświadczam ponadto, że niniejsza wersja pracy jest identyczna z załączoną wersją elektroniczną.

Data 08. 03. 2022 r. Podpis autora pracy A. Matuszewski

Spis treści

Streszczenie

Nanobiotechnologia jest stosunkowo nową, aczkolwiek bardzo intensywnie rozwijającą się dziedziną nauki dotyczącą tworzenia i wykorzystania struktur o wielkości mniejszej niż 100 nm - nanocząstek i nanomateriałów. W aspekcie wysokich wskaźników produkcyjnych kurcząt brojlerów należy pamiętać o problemie słabego kośćca jako efektu ubocznego hodowli w kierunku maksymalizacji produkcji. Stale poszukuje się nowych rozwiązań, które mogłyby okazać się efektywne w poprawie jakości kości kurcząt. Potencjalną metodą jest podawanie związków drogą iniekcji bezpośredniej do jaja (*in ovo*), które wykazują powinowactwo do tkanki kostnej. Takimi związkami mogą być nanometryczne formy węglanu wapnia i hydroksyapatytu. Wpływając na jakość kości przyczyniają się do poprawienia komfortu ptaków, ograniczając ryzyko wystąpienia schorzeń nóg. Badania podzielono na dwa główne panele doświadczeń, których celem były:

a) ocena wpływu różnych stężeń nanohydroksyapatytu podanych *in ovo* do białka na rozwój zarodka kury ze szczególnym uwzględnieniem rozwoju układu kostnego,
b) ocena wpływu wybranego stężenia nanocząstek węglanu wapnia podanych *in ovo* do białka na rozwój zarodka kury, a także na wyniki produkcyjne, jakość mięsa i jakość kości kurcząt brojlerów w 42. dniu życia.

Panel dodatkowy stanowiła ocena przeżywalności i stopnia mineralizacji ptasich komórek osteogennych po ekspozycji na rosnące stężenie nanocząstek węglanu wapnia.

Badane nanocząstki podane drogą iniekcji bezpośredniej do białka jaj nie wykazywały negatywnego wpływu na przeżywalność zarodków i ich rozwój. Wykazano zależny od rodzaju nanocząstek wpływ na modulację efektu kostnego i status redox. Wykazano, że bardziej korzystny wpływ na zarodek mają nanocząstki węglanu wapnia. Węglan wapnia w formie nanometrycznej, podany *in ovo* do białka wpłynął pozytywnie na jakość kości kurcząt brojlerów, nie pogarszając ich wskaźników produkcyjnych, wyników analizy rzeźnej. Badania *in vitro* wykazały pozytywny wpływ nanocząstek węglanu wapnia na żywotność i mineralizację ptasich komórek osteogennych.

Summary

Nanobiotechnology is a relatively new, but very intensively developing field of science concerning the use, creation and characterization of materials smaller than 100 nm - nanoparticles and nanomaterials. In the aspect of high production rates of broiler chickens, it is important to remember about the problem of weak bones as a side effect of breeding towards maximizing production. New solutions that could be effective in improving the quality of chicken bones are constantly being sought. A potential approach is to administer the compounds in various forms which show an affinity for bone tissue by direct injection into the egg (*in ovo*). Such compounds can be nanometric forms of calcium carbonate and hydroxyapatite By affecting the quality of the bones, they contribute to the improvement of bird comfort, reducing the risk of leg diseases. The research was divided into two main panels of experiments, the aim of which was to:

a) assess the effect of various concentrations of nanohydroxyapatite administered *in ovo* to the albumen on the development of the chicken embryo, with particular emphasis on the development of the skeletal system.
b) evaluate the effect of the selected concentration of calcium carbonate nanoparticles administered *in ovo* to the albumen on the development of the chicken embryo, as well as on production results, slaughter analysis and meat and bone quality of broiler chickens on the 42 day of life.

The additional panel was an assessment of the livability and mineralization degree of avian osteogenic cells after exposure to increasing concentrations of calcium carbonate nanoparticles.

Differences in the interaction of both types of nanoparticles with the chicken embryo model were demonstrated. The nanoparticles showed no negative impact on the survival of the embryos and their development. Due to different physicochemical properties, their modulating effect on embryo bone turnover or redox status was different. Calcium carbonate nanoparticles have been shown to have a more beneficial effect on the embryo. Moreover, calcium carbonate administered *in ovo* to the albumen had a positive effect on the bone quality of broiler chickens without deteriorating their production results, slaughter analysis results. In vitro studies have shown a positive effect of calcium carbonate nanoparticles on the viability and mineralization of avian osteogenic cells.

Wykaz publikacji stanowiących rozprawę doktorską pod tytułem:

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Rozprawa doktorska obejmuje trzy publikacje naukowe – jedną o charakterze przeglądowym i dwie prace eksperymentalne.

Matuszewski A, Łukasiewicz M, Niemiec J. 2020. Calcium and phosphorus and their nanoparticle forms in poultry nutrition. *World's Poultry Science Journal*, 76 (2): 328-345. Doi:10.1080/00439339.2020.1746221 **(IF 2,915 , 70 pkt., cyt. Wg Wos: 3)**.

Matuszewski A, Łukasiewicz M, Niemiec J, Jaworski S, Kamaszewski M, Szudrowicz H, Puppel K, Chwalibog A, Sawosz E. 2020. Effect of in ovo application of hydroxyapatite nanoparticles on chicken embryo development, oxidative status and bone characteristics. *Archives of Animal Nutrition*, 74(5): 343-362. Doi: 10.1080/1745039X.2020.1803033 **(IF 2,242, 100 pkt., cyt. wg WoS: 1)**.

Matuszewski A, Łukasiewicz M, Niemiec J, Kamaszewski M, Jaworski S, Domino M, Jasiński T, Chwalibog A, Sawosz E. 2021. Calcium Carbonate Nanoparticles—Toxicity and Effect of In Ovo Inoculation on Chicken Embryo Development, Broiler Performance and Bone Status. *Animals*, 11(4): 932. Doi: 10.3390/ani11040932 (**IF 2,752, 100 pkt., cyt. wg WoS: 1**).

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Wykaz stosowanych skrótów:

BALP – frakcja kostna fosfatazy alkalicznej

Ca – wapń

CCN – nanocząstki węglanu wapnia

FCR – współczynnik wykorzystania paszy

GR – reduktaza glutationowa

GSH – glutation

GSH-PX – peroksydaza glutationowa

Hnp.NP – nanocząstki hydroksyapatytu

MDA – dialdehyd malonowy

OC – osteokalcyna

OPG – osteoprotegeryna

P – fosfor

PINP – N – końcowy propeptyd prokolagenu typu I

SOD – dysmutaza ponadtlenkowa

TAS – całkowity status antyoksydacyny

1. Wstęp

Nanobiotechnologia to dziedzina nauki dotycząca struktur, które w co najmniej jednym wymiarze cechują się rozmiarem mniejszym od 100 nm. Nanocząstki są znacznie mniejsze od swoich odpowiedników w skali makrometrycznej, tym samym wzrasta ich stosunek powierzchni do objętości. To powoduje, że obiekty w skali nanometrycznej mogą posiadać unikalne właściwości optyczne, chemiczne i fizyczne. Nanomateriały, a w tym nanocząstki otwierają nowe możliwości badawcze i aplikacyjne. Jedną z gałęzi, w której możliwości zastosowania nanocząstek są znaczące, jest produkcja drobiarska (Fisinin i wsp., 2018; Abd El-Ghany, 2019). Ostatnie 10 lat to przełom jeżeli chodzi o badania nad wykorzystaniem nanocząstek w chowie i hodowli drobiu. Obecne prace zespołów badawczych skupiają się przede wszystkim nad wykorzystaniem pierwiastków w formie nanometrycznej jako zamienników dla ich tradycyjnych źródeł (Ramiah i wsp., 2019), jako alternatywy dla antybiotyków (Pineda i wsp., 2012) i stymulatorów wzrostu (Andi i wsp., 2011). Nie bez znaczenia pozostaje aspekt pośredni, jakim jest wpływ produkcji zwierzęcej na środowisko. Zastosowanie nanocząstek, które charakteryzują się mniejszym rozmiarem, wyższą reaktywnością i efektywniejszą przyswajalnością, stwarza możliwości do ograniczania ilości stosowanych pierwiastków w takiej formie. Nanocząstki srebra (Sawosz i wsp., 2012; Chauke i Siebrits, 2012; Sawosz i wsp. , 2013; Ahmadi i wsp., 2013; Elkloub i wsp., 2015), selenu (Fuxiang i wsp., 2008; Wang, 2009; Zhou i Wang, 2011; Cai i wsp., 2012; Mohapatra i wsp., 2014; Selim i wsp., 2015; Shirsat i wsp., 2016), miedzi (Wang i wsp., 2011; Pineda i wsp., 2013; Mroczek-Sosnowska i wsp., 2015, 2017; Sawosz i wsp., 2018; Sizova i wsp., 2019; Łukasiewicz i wsp., 2020), manganu (Matuszewski i wsp., 2020), jak również ich kombinacje w innymi składnikami odżywczymi (Yausheva i wsp., 2016) można zaliczyć do głównych obszarów zainteresowań w kontekście zastosowania w produkcji drobiarskiej.

Nanocząstki związków wapnia, będące kolejną, ważną grupą związków,mają także perspektywiczne znaczenie w wykorzystaniu w chowie i hodowli drobiu (Matuszewski i wsp., 2020a). W tej grupie należy wskazać fosforan jedno – i dwuwapniowy, węglan wapnia oraz hydroksyapatyt. Suplementacja nanocząstkami związków wapnia ma na celu ograniczenie stosowania tradycyjnych źródeł wapnia

i fosforu w paszy, co skutkuje zmniejszeniem kosztów suplementacji. Wysokie powinowactwo nanocząstek wapniowych do tkanki kostnej skłania również do określania ich wpływu na układ kostny ptaków. Większość dotychczasowych doniesień traktuje o wpływie podawania nanocząstek związków wapnia drogą *per os* w wodzie albo w paszy. W badaniach uwzględniających nanocząstki fosforanu wapnia (Vijayakumar i Balakrishnan, 2014; Vjayakumar i Balakrishnan, 2015; Samanta i wsp., 2019) oraz fosforanu dwuwapniowego (Hassan i wsp., 2016; Mohamed i wsp., 2016) głównym założeniem był aspekt ograniczania stosowania dużych ilości wybranych źródeł pierwiastków, poprzez zastosowanie form o większej biodostępności. Pierwsze doniesienia skupiały się na wpływie nanocząstek fosforanu wapnia na organizm ptaka. Wskaźniki biochemiczne krwi takie jak glukoza, albuminy, trójglicerydy, cholesterol, kreatynina, czy wybrane enzymy wątrobowe nie uległy istotnym, negatywnym zmianom przy zastosowaniu fosforanu wapnia w formie nanometrycznej w porównaniu do kurcząt żywionych tradycyjnym fosforanem dwuwapniowym. Podobnie przedstawiały się wybrane parametry hematologiczne (Vijayakumar i Balakrishnan, 2015). W badaniach Mohamed i wsp. (2016) nie wykazano istotnych zmian jeżeli chodzi o procentowy udział narządów takich jak żołądek, serce i wątroba określanych w trakcie analizy rzeźnej kurcząt brojlerów. Zastosowanie w diecie tylko 25% wymaganego fosforu niefitynowego pochodzącego z nanocząstek fosforanu dwuwapniowego mogło być zamienne dla 100% zapotrzebowania na ten pierwiastek pochodzący z formy tradycyjnej w paszy. Ci sami autorzy zaobserwowali wyższą końcową masę ciała ptaków, lepszą wartość współczynnika wykorzystania paszy i większe dzienne przyrosty masy ciała z różnych grup żywionych paszami z udziałem fosforanu dwuwapniowego w formie nanometrycznej. Zaobserwowano zmniejszone wydalanie Ca i P w kale przez ptaki z grup z udziałem nanocząstek o około 50% (Hassan i wsp., 2016). Vijayakumar i Balakrishnan (2015) wykazali brak istotnych różnic w morfometrii i mineralizacji kości piszczelowej przy zastosowaniu w 100% fosforanu dwuwapniowego (grupa kontrolna) jako źródła Ca i P oraz rosnących procentowych udziałów nanocząstek fosforanu wapniowego zamiast formy standardowej (od 50 do 100% formy nanometrycznej). Potwierdziło to, że biodostępność fosforu i wapnia z formy nanometrycznej fosforanu wapniowego jest o 200% skuteczniejsza w porównaniu do formy tradycyjnej. U kurcząt żywionych paszą z dodatkiem nanohydroksyapatytu również stwierdzono wyższą masę

ciała oraz mniejsze spożycie paszy przez ptaki. Dodatkowo zaobserwowano lepszą przyswajalność Ca i P z paszy zawierającej hydroksyapatyt w formie nanometrycznej. W badaniach Mohamed i wsp. (2016) porównano wpływ nanocząstek fosforanu dwuwapniowego i fosforanu dwuwapniowego w formie tradycyjnej na jakość kości długich 26. dniowych kurcząt brojlerów. Interesujący jest fakt, że zastosowane nanocząstki fosforanu dwuwapniowego wpłynęły pozytywnie na parametry kości piszczelowej takie jak masa, długość, szerokość i siła łamania, przy zastosowaniu ich w znacznie mniejszej dawce w porównaniu do ich tradycyjnego odpowiednika. Węglan wapnia w formie nanometrycznej był oceniany pod kątem jego przydatności w żywieniu kur nieśnych. W badaniach Ganjigohari i wsp. (2018a, 2018b) wykazano, że istnieje możliwość zmniejszenia poziomu węglanu wapnia w formie nanocząstek od 0,252 do 2,015% zamiast 4,03% dla tradycyjnej kredy pastewnej, przy jednoczesnym braku obniżenia masy jaj i pogorszenia wykorzystania paszy (FCR). Warto przy tym wskazać, że redukcja ilości nano węglanu do poziomu 0,126% (przy zachowaniu 4,03% kredy pastewnej) wpłynęła na pogorszenie parametrów produkcyjnych kur, wybranych wyróżników jakości jaj, grubości kości piszczelowej i Ca we krwi, na skutek zbyt małej podaży Ca w paszy. Z kolei wykorzystanie naturalnie syntetyzowanych CCN w rosnących udziałach (od 0 do 1,5g/kg) w połączeniu z ekstraktem z alg w paszy dla kur niosek wpłynęło na poprawienie parametrów jakościowych jaj, takich jak grubość skorupy, masa skorupy, masa skorupy w przeliczeniu na jednostkę powierzchni, a także na wzrost poziomu wolnego Ca i P we krwi ptaków (El-Maaty i wsp., 2020). Obiecująca perspektywa wykorzystania nanocząstek związków wapnia w tradycyjnym żywieniu drobiu, wymaga jednak dalszych badań począwszy od wpływu nanocząstek na organizm ptaka, ich toksyczności, bioakumulacji w organizmie, a kończąc na ustaleniu optymalnych dawek dla poszczególnych grup ptaków produkcyjnych.

Technologia *in ovo*, pomimo, że znana i stosowana w praktyce drobiarskiej już prawie 30 lat (lata 90. XX wieku to czas wdrożenia automatyzacji szczepienia *in ovo* przeciwko chorobie Mareka w USA), nadal stanowi interesującą metodę tak zwanego odżywiania funkcjonalnego (dożywiania). Nowoczesne linie kurcząt brojlerów są selekcjonowane w kierunku zwiększonego tempa wzrostu, wyższej końcowej masy ciała, wyższego udziału mięśni piersiowych w tuszce, co skutkuje wyższymi wymaganiami zarodka dotyczącymi białka i energii, czy rezerw składników mineralnych (Ghobadi i Martin, 2015). Suplementacja składnikami

mineralnymi już na etapie embriogenezy, ma duże znaczenie zwłaszcza dla piskląt brojlerów o wysokim potencjale produkcyjnym. Badania dotyczące wpływu różnych substancji aplikowanych *in ovo* na rozwój zarodka czy też status piskląt po wylęgu są liczne i prezentują ciekawe rezultaty. Iniekcja do jaja aminokwasów, związków mineralnych, węglowodanów, ekstraktów roślinnych, czy witamin pokazuje, że ta metoda dożywiania może pozytywnie wpływać na przeżywalność zarodków, ich status immunologiczny, morfologię jelit, czy masę narządów (Selim i wsp., 2012; Gonzales i wsp., 2013; Yair i wsp., 2013; Saki i Salary, 2015; Zhang i wsp., 2018). Biorąc pod uwagę wysokie wskaźniki produkcyjne należy pamiętać o efektach ubocznych hodowli w kierunku maksymalizacji produkcji, jak na przykład słaby kościec. Stale poszukuje się nowych rozwiązań, które mogłyby okazać się efektywne w poprawie jakości kości kurcząt. Potencjalną metodą jest podawanie związków w różnych formach, które wykazują powinowactwo do tkanki kostnej, drogą iniekcji bezpośredniej do jaja. Za przykład mogą posłużyć wymienione powyżej nanomateriały takie jak CCN czy HA-NP. Danych literaturowych dotyczących wpływu nanocząstek związków wapnia podanych *in ovo* na rozwój układu kostnego zarodka kury, czy w późniejszym okresie – kurcząt, jest niewiele. W badaniach Ahmadzadeh i wsp. (2019) porównujących wpływ HA-NP powstałego w wyniku syntezy bakteryjnej oraz syntezy chemicznej na rozwój zarodka kury, nie wykazano wpływu na przeżywalność zarodków i ich masę ciała. Dodatkowo stwierdzono lepszą skuteczność w zwiększeniu mineralizacji i gęstości mineralnej kości zarodkównp.la HA-NP z syntezy bakteryjnej. Pierwsze doświadczenia dotyczące iniekcji *in ovo* CCN, nie wykazały ich negatywnego wpływu na przeżywalność zarodków kury, jednocześnie zwiększając depozyt Ca w kości piszczelowej (Salary i wsp., 2017). W tym miejscu warto wspomnieć o udowodnionym wpływie nanocząstek miedzi podanych *in ovo* na masę i długość kości u kurcząt brojlerów (Mroczek-Sosnowska i wsp., 2017), kompleksu minerałów, witamin A, D_3, E i maltodekstryny na polepszenie mineralizacji, struktury i właściwości mechanicznych kości zwłaszcza pod koniec okresu embrionalnego i u kurcząt brojlerów (Yair i wsp., 2013), zaś cynku, manganu i miedzi pochodzenia organicznego na zwiększenie zawartości popiołu w kościach (*de facto* mineralizacji) (Oliveira i wsp., 2015). Stwierdzić zatem można, że potencjał aplikacyjny zastosowania nanomateriałów i innych związków *in ovo* celem

polepszenia jakości kości u zarodków kury oraz osiągnięcia dalekosiężnych efektów w postaci poprawy kośćca, jest zasadny. Ten obszar badań wciąż jednak wymaga uzupełnienia o szereg informacji, takich jak np. toksyczność nanocząstek, czy wyjaśnienie mechanizmu ich oddziaływania na zarodek kury. Badania w obszarze zastosowania nanotechnologii w biotechnologii drobiu, jako źródła łatwo przyswajalnych minerałów podawanych *in ovo* i mogących wpłynąć na rozwój układu lokomotorycznego zarodka, a następnie kurcząt są ważne z tego powodu, że mogą przyczynić się do polepszenia komfortu ptaków (Matuszewski i wsp., 2020a). Ponadto straty ekonomiczne wywoływane przez różne schorzenia systemu szkieletowego kurcząt brojlerów są dziś duże i sięgają nawet 120 milionów USD w Stanach Zjednoczonych (Cook, 2000). Według badań brytyjskich naukowców, nawet ok. 27% ptaków w stadach kurcząt jest dotkniętych schorzeniami układu kostno-szkieletowego (Knowles i wsp., 2008). Niewątpliwie jest to problem, z którym mierzą się producenci drobiu.

2. Hipoteza badawcza, cel i zakres pracy

W ramach pracy doktorskiej postawiono następujące hipotezy badawcze:

- Nanocząstki hydroksyapatytu, będące nietoksycznym suplementem, podane *in ovo* do białka na początku inkubacji nie wpływają negatywnie na rozwój zarodka kury. Nanocząstki hydroksyapatytu ze względu na rozmiar są łatwo wchłaniane przez komórki zarodka, działając stymulująco i modulująco, szczególnie w odniesieniu do rozwoju układu kostnego.

- Nanocząstki węglanu wapnia będące nietoksycznym suplementem, podane *in ovo* do białka w wywierają szereg efektów stymulujących i modulujących rozwój zarodka kury oraz wpływają na jakość kości kurcząt brojlerów bez pogarszania ich wskaźników produkcyjnych. Nanocząstki węglanu wapnia, jako zewnętrzne źródło łatwo przyswajalnego Ca wpływają na regulację osteokalcyny – białka odpowiedzialnego za wiązanie hydroksyapatytu, co skutkuje zwiększeniem mineralizacji kości.

Celem przeprowadzonych, w ramach pracy doktorskiej, badań było określenie wpływu podawanych nanocząstek wybranych związków wapnia drogą iniekcji bezpośredniej do białka jaja na rozwój zarodka kury, wyniki produkcyjne i układ lokomotoryczny kurcząt brojlerów w 42. dniu życia.

Zakres badań:

1) Określenie toksyczności, dawkozależności nanocząstek z wykorzystaniem ptasich komórek osteogennych w badaniach *in vitro* poprzez określenie żywotności komórek oraz zbadanie ich stopnia mineralizacji.
2) Określenie wpływu HA-NP i CCN w różnych stężeniach podanych *in ovo* do białka na przeżywalność oraz masę zarodków i ich narządów, status redoks, status zdrowotny, morfometrię i mineralizację kości długich, zawartość wybranych markerów obrotu kostnego w tkankach.
3) Określenie wpływu CCN podanych *in ovo* do białka na parametry produkcyjne, wyniki analizy rzeźnej, jakość mięśnia piersiowego, morfometrię i mineralizację kości udowej, zawartość wybranych markerów obrotu kostnego w tkankach, obraz histologiczny kości.

3. Metodyka badań

3.1. Nanocząstki związków wapnia

W badaniach wykorzystano dwa rodzaje nanocząstek – nanocząstki hydroksyapatytu (HA-NP) i nanocząstki węglanu wapnia (CCN). Obydwa rodzaje nanocząstek zostały zakupione w Skyspring Nanomaterials (Houston, Teksas, Stany Zjednoczone). Nanocząstki miały formę białych proszków. Proszki zawieszano w ultraczystej wodzie (o przewodności 18,3 MΩ x cm, mierzonej w temperaturze 25^{o}C) i poddawano sonikacji przez 45 min. w celu pozyskania roztworów koloidalnych. Wielkość oraz kształt nanocząstek określano poprzez wizualizację z wykorzystaniem transmisyjnego mikroskopu elektronowego Morgagni 268D z kamerą szerokokątną Olympus Morada (FEI, Oregon, Stany Zjednoczone). Ponadto zbadano potencjał Zeta hydrokoloidów nanocząstek wykorzystując analizator

Zetasizer Nano-ZS90 (Malyern, Worcestershire, Wielka Brytania). W tabeli 1. przedstawiono wybrane właściwości fizykochemiczne nanocząstek.

Tabela 1. Porównanie właściwości fizykochemicznych nanocząstek hydroksyapatytu i węglanu wapnia zastosowanych w badaniach (Matuszewski i wsp., 2020b; Matuszewski i wsp., 2021).

Cecha	**Nanocząstki**	
	hydroksyapatytu HA-NP	**węglanu wapnia CCN**
Czystość	> 99,5%	> 97,5%
Wielkość pojedynczych nanocząstek	20-35 nm	15-40 nm
Kształt	kulisty	sześcienny
Potencjał zeta	-12,3 mV	-20 mV
Zdolność do aglomeracji	aglomeraty ok. 400 nm	aglomeraty > 1000 nm
Metoda produkcji	redukcja chemiczna	redukcja chemiczna

3.2. Modele badawcze

3.2.1. Ptasie komórki osteogenne

W pracy wykorzystano komórki ptasie wyizolowane z kości udowej zarodka kury. W 12. dniu inkubacji zarodki zostały uśmiercone, a następnie wypreparowano z nich kość udową. Do izolacji komórek osteogennych wykorzystano zmodyfikowaną metodę Li i wsp. (2015). Z uprzednio oczyszczonych z tkanek miękkich kości pobrano nasadę większą, rozdrobniono mechanicznie, a następnie traktowano kolagenazą przez 30 min. Zawiesinę przefiltrowano, a następnie wirowano przez 10 minut. Płyn nad osadem odsączono, a sam osad został zawieszony na pożywce hodowlanej Dulbecco zmodyfikowanej przez Eagle'a (ang. Dulbecco's modified Eagle's culture medium; DMEM) (Sigma-Aldrich®, St. Louis, USA) wzbogaconej 10% cielęcą surowicą płodową (Sigma-Aldrich®) z dodatkiem 1% penicyliny i streptomycyny (Sigma-Aldrich®). Linie komórkowe hodowane były w plastikowych naczyniach przeznaczonych do hodowli komórek adherentnych. Hodowle prowadzone były w inkubatorze do hodowli komórkowych

(NuAir, Minessota, Stany Zjednoczone) zapewniającym stałe warunki temperatury i dwutlenku węgla (temperatura 37 ^{o}C, CO_2 5%).

3.2.2. Zarodek kury

Do badań *in ovo* użyto jaj wylęgowych pochodzących od stad rodzicielskich kurcząt Ross 308. Jaja początkowo były przechowywane w chłodni w 12^{o}C i wilgotności 73% przez okres dwóch dni. Wstępnie ogrzane zostały umieszczone w aparacie lęgowym (Jamesway, Cambridge, Kanada). Standardowe warunki inkubacji wynosiły 37,8^{o}C i wilgotności 55% (zwiększonej do 75% pod koniec inkubacji). W trakcie inkubacji jaja były automatycznie obracane raz na godzinę. Prześwietlanie jaj następowało w 7. i 18. dobie inkubacji celem odrzucenia jaj niezapłodnionych bądź z zamarłym zarodkiem. Jaja przed wstawieniem do aparatu zostały poddawane kąpielom w 0,5% roztworze nadmanganianu potasu. Jaja z grup doświadczalnych były nastrzykiwane hydrokoloidami nanocząstek wybranych związków wapnia w dniu zerowym do białka z wykorzystaniem strzykawek z igłą 27G. Otwór w jaju został zaklejony plastrem hipoalergicznym natychmiast po wprowadzeniu 0,2 ml koloidu. W 20. dniu inkubacji zarodki były ważone, a następnie uśmiercane. W trakcie dekapitacji pobierano krew na skrzep do szklanych probówek, zaś potem wybrane narządy (po schłodzeniu na lodzie) do dalszych analiz. Materiał biologiczny był przechowywany w temperaturze -80^{o}C.

3.2.3. Kurczęta brojlery

Kurczęta linii Ross 308 pochodziły z jaj inkubowanych jak w podrozdziale 3.2.2., pozyskanych z lokalnego zakładu wylęgowego. Pisklęta z jaj z grupy kontrolnej (bez iniekcji) oraz grupy doświadczalnej (po iniekcji *in ovo* do białka) po wykluciu zostały dostarczone na Fermę Doświadczalną Wilanów-Obory SGGW. Pierwszego dnia pisklęta zaszczepiono przeciwko chorobie Mareka, Gumboro i zakaźnemu zapaleniu oskrzeli (IB), a następnie 15. dnia przeciwko Gumboro i IB. Następnie pisklęta losowo przydzielono do boksów (według grup badawczych), których powierzchnia (kg/m^2) była zgodna z instrukcją prowadzenia stada dla kurcząt linii Ross 308. Ptaki utrzymywano na sieczce słomianej pszennej w standardowych warunkach tj. temperatura 32^{o}C w pierwszym tygodniu, kolejno obniżana o ok. 2^{o}C tygodniowo do 20^{o}C w ostatnim tygodniu. Wilgotność powietrza

wynosiła 60%, zastosowano 24-godzinny program światła. W trakcie odchowu, w cotygodniowych odstępach, kontrolowano parametry mikroklimatu takie jak wilgotność i stężenie gazów (amoniaku, dwutlenku węgla, siarkowodoru) Ptaki miały zapewniony stały dostęp do paszy i wody. Zastosowano trzyfazowy system żywienia z zastosowaniem mieszanek pełnoporcjowych typu starter (1-10. dzień), grower (11-34. dzień) i finiszer (35-42. dzień) (tab. 2). Kurczęta odchowywano do 42. dnia życia, kontrolując masę ciała w dniach zmian mieszanek (1., 10., 35., 42.).Na koniec odchowu określono wskaźniki produkcyjne kurcząt tj. przyrost masy ciała (g), śmiertelność (%), współczynnik wykorzystania paszy (kg/kg). W trakcie uboju ptaków pobrano krew (na skrzep) do dalszych analiz. Wypatroszone tuszki schłodzono w 4^{o}C przez 24h, a następnie przeprowadzono dysekcję według metody Ziołeckiego i Doruchowskiego (1989). Określono wydajność rzeźną, procentowy udział mięśni piersiowych i nóg oraz podrobów. Przeznaczone do dalszych analiz mięśnie piersiowe (shomogenizowane) przechowywano w temperaturze -20^{o}C. Wypreparowane w trakcie dysekcji kości udowe oraz odwirowaną z uprzednio pobranej krwi surowicę przechowywano w -80^{o}C z przeznaczeniem do dalszych oznaczeń. Doświadczenia na kurczętach brojlerach i zarodkach wykonane zostały za zgodą Lokalnej Komisji Etycznej (pozwolenie nr 46/2015).

Tabela 1. Komponenty i skład chemiczny mieszanek paszowych dla kurcząt brojlerów (Matuszewski i wsp., 2021).

Komponent (g/kg)	**Starter**	**Grower**	**Finiszer**
Kukurydza	100	114	100
Pszenica	530	550	608
Śruta poekstrakcyjna sojowa	306	274	216
Węglan wapnia	11,9	12	9,7
Wodorowęglan sodu	2	1,4	1,6
Stymulator	0,1	0,1	0,1
Fosforan dwuwapniowy	11,8	7,8	6,4
Olej	21	24	44
Metionina 84%	4,8	4,2	2,8
Lizyna	3,6	3,4	2,8
Treonina	1,4	1,3	1
Premiks	5	5	5
		Skład chemiczny g/kg	
Białko surowe	219	207	187
Tłuszcz surowy	47,1	52,3	68,1
Popiół	50,7	51,3	51,7
Lizyna	12,8	11,8	11,1
Metionina	7,2	6,3	5,4
Wapń	9,5	7	5,5
Fosfor	6,6	5,3	5,1
Energia metaboliczna(MJ/kg)	12,28	12,54	12,75

3.3. Układ doświadczeń

Przeprowadzono dwa główne panele doświadczeń, których celem była:

I. Ocena wpływu różnych stężeń HA-NP aplikowanych *in ovo* do białka na rozwój zarodka kury, ze szczególnym uwzględnieniem rozwoju układu kostnego (Matuszewski i wsp., 2020b) (rys. 1).

II. Ocena wpływu wybranego stężenia CCN podanego *in ovo* do białka na rozwój zarodka kury a także na wyniki produkcyjne, jakość mięsa i kości kurcząt brojlerów w 42. dniu życia (Matuszewski i wsp., 2021) (rys. 2).

Przeprowadzono panel dodatkowy, którego celem była:

Ocena żywotności i stopnia mineralizacji ptasich komórek osteogennych po ekspozycji na rosnące stężenie CCN (Matuszewski i wsp., 2021).

Panel I: Ocena wpływu różnych stężeń HA-NP aplikowanych *in ovo* do białka na rozwój zarodka kury, ze szczególnym uwzględnieniem rozwoju układu kostnego (Matuszewski i wsp., 2020b).

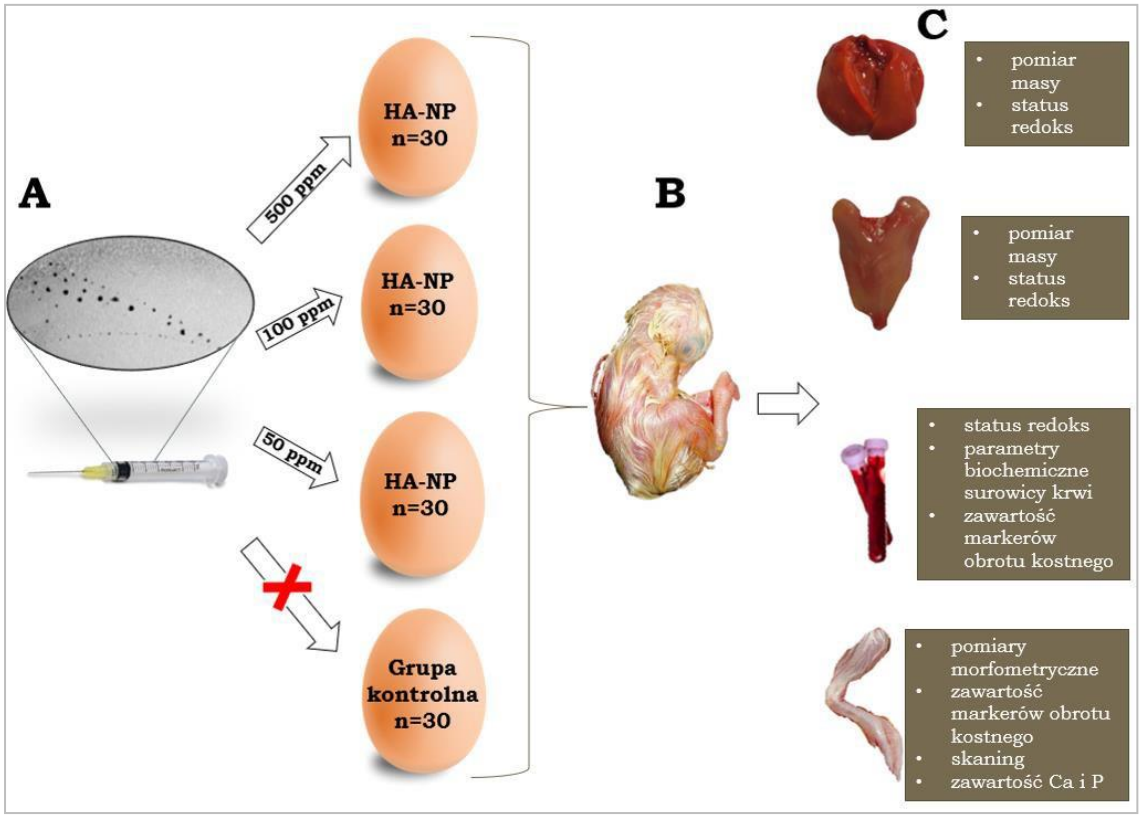
Materiał doświadczalny stanowiło 120 jaj pochodzących ze stada rodzicielskiego kur linii Ross 308. Każde z jaj przed nałożeniem do aparatu zostało zważone i losowo podzielone do jednej z 4 grup (po 30 jaj w każdej). Grupę kontrolną stanowiły jaja nienastrzykiwane. Trzy grupy eksperymentalne różniło stężenie roztworu koloidalnego HA-NP: 50, 100 oraz 500 μg/ml. Iniekcja do jaja przeprowadzona została według metody opisanej w podrozdziale 3.2.2. W układzie doświadczenia celowo zrezygnowano z uwzględnienia tak zwanej grupy kontrolnej pozornej (poddanej iniekcji PBS), ze względu na dużą zamieralność zarodków. W 20. dniu inkubacji zarodki zostały zważone i uśmiercone. W trakcie dekapitacji pobrano 10 prób krwi z tętnicy szyjnej (próbę stanowiła krew zbiorcza z dwóch zarodków) dla każdej z grup. Krew została natychmiast schłodzona w temperaturze 4°C przez dwie godziny, a następnie wirowana przez 5 min z prędkością 1200 obrotów/min. (wirówka MPW-350R, MPW Med. Instruments, Polska). Ze każdej grupy losowo wybrano po 10 zarodków, z których wyizolowano i zważono mięśnie piersiowe, wątrobę, kości udową oraz piszczelową. Procent przeżywalności zarodków stanowił stosunek żywych zarodków w 20. dobie inkubacji do liczby zapłodnionych jaj w danej grupie.

W surowicy oznaczono podstawowe parametry biochemiczne krwi takie jak AST, ALT, ALP LDH, glukozę, kreatyninę, białko całkowite, albuminy, cholesterol całkowity, trójglicerydy, Ca i P z użyciem zestawów komercyjnych w Weterynaryjnym Laboratorium Diagnostycznym SGGW w Warszawie. Ponadto, w surowicy określono zawartość MDA z zastosowaniem metody Kapusty i wsp. (2018) bazującej na reaktywności z kwasem tiobarbiturowym powstających produktów ubocznych peroksydacji lipidów. Określono również koncentrację GSH bazując na metodzie Ellmana, zmodyfikowanej przez Matusiewicz i wsp. (2019), w której kwas 5,5'-ditiobis-(2-nitrobenzesowy) zredukowano przez związki tiolowe do otrzymania barwnego produktu. W obydwóch metodach wybrane markery statusu redoks oznaczano kolorymetrycznie, a absorbancję odczytywano na analizatorze mikropłytek Tecan's NanoQuant Infinite M200 PRO (Tecan, Grödig,

Austria). W tkankach miękkich tj. mięsień piersiowy i wątroba oznaczono aktywność markerów oksydacyjnych – SOD, TAS, GSH-PX i GR. Sześć prób obu tkanek dla każdej z 4 grup, o odważonej masie ok. 200 µg umieszczono w 2 ml probówce typu Eppendorf, a następnie dodano 1 ml PBS. Całość homogenizowano. Poziom białka w próbie oznaczono przy użyciu kitu z kwasem bicynchoninowym (BCA, Merck, Warszawa, Polska). 25 µg homogenatów przenoszono na mikropłytkę, dodając kolejno odczynniki z kitów do oznaczania wyżej wymienionych markerów (Randox, Polska). Odczyt przeprowadzono na spektrofotometrze płytkowym Tecan NanoQuant Infinite M200 Pro. Wyniki były przeliczane względem wystandaryzowanej ilości białka w próbie.

Wyizolowane z zarodków kości (udowa i piszczelowa) w liczbie 10 sztuk prawych i 10 sztuk lewych, dla obu rodzajów kości w każdej z grup zostały oczyszczone, a następnie zmierzono ich długość, średnicę na środku trzonu oraz średnicę nasady bliższej używając suwmiarki elektronicznej (Mitutoyo, Wrocław, Polska) z dokładnością do dwóch miejsc po przecinku. Wszystkie kości zostały poddane analizie wytrzymałości określając tak zwaną siłę łamania (maszyna wytrzymałościowa Zwick Z 0.5, Zwicki-Line, Ulm, Niemcy). Odcinki nasady bliższej kości udowej zostały poddane obrazowaniu mikroarchitektury za pomocą skaningowego mikroskopu elektronowego (FEI, Hillsboro, Stany Zjednoczone). Sześć lewych kości udowych zostało zmiażdżonych w moździerzu, następnie homogenizowano je w 1 ml PBS. Wykonano również oznaczenie ilościowe białka wg. metody podanej powyżej. W homogenatach oznaczono wybrane markery obrotu kostnego – osteokalcynę (OC) oraz N-końcowy propeptyd prokolagenu typu I (PINP) wykorzystując do tego testy immunoenzymatyczne ELISA (Immunogen, Warszawa, Polska), specyficzne dla gatunku kura domowa *Gallus gallus domesticus.* W wyizolowanej surowicy (10 prób dla każdej grupy) również przeprowadzono oznaczenia wybranych markerów obrotu kostnego – OC, PINP, osteoprotegeryny (OPG) oraz frakcji kostnej fosfatazy alkalicznej (BALP). Odczyty absorbancji prowadzono na analizatorze Tecan NanoQuant Infinite M200 Pro. Sześć prawych kości udowych i piszczelowych oddymiono na płycie grzejnej i spalono w piecu muflowym w temperaturze ok 470°C przez 36 h. Po ostudzeniu do próbek dodano po 2 ml wody redestylowanej i HCl (38%) i przeniesiono ilościowo za pomocą wody redestylowanej do 40 ml. Analizy Ca i P dokonano przy użyciu spektrometru emisji

atomowej ICP-AES Thermo iCAP 6500 DUO (Thermo Fisher Scientific, Waltham, Massachusetts, Stany Zjednoczone). Metoda polega na pomiarze w roztworach badanych próbek intensywności emitowanego promieniowania (charakterystycznego dla danego pierwiastka), która jest miarą stężenia oznaczonego pierwiastka. Schematyczny układ doświadczenia 1 przedstawiono na rys. 1.



Rys. 1. Schemat doświadczenia 1. Objaśnienie skrótów: A – iniekcja 200 µl hydrokoloidu HA-NP w różnych stężeniach do białka jaja; B – pobranie zarodków w 20. dobie inkubacji, izolacja wątroby, mięśni piersiowych, krwi, kości kończyny dolnej; C – wykonywane analizy na poszczególnych tkankach i narządach.

II. Ocena wpływu wybranego stężenia CCN podanego *in ovo* do białka na rozwój zarodka kury a także na wyniki produkcyjne, jakość mięsa i kości kurcząt brojlerów w 42. dniu życia (Matuszewski i wsp., 2021).

Materiał doświadczalny stanowiło 240 jaj wylęgowych pochodzących od kur stad rodzicielskich brojlerów Ross 308. Przed rozpoczęciem inkubacji, jaja były utrzymywane w takich samych warunkach, jak w doświadczeniu I. Jaja podzielono

na dwie grupy, po 120 szt. w każdej. Grupę kontrolną stanowiły jaja niepoddawane manipulacjom, zaś grupę doświadczalną jaja, które zostały poddane iniekcji do białka 500 μg/ml stężeniem roztworu hydrokoloidalnego CCN. W układzie doświadczenia celowo zrezygnowano z uwzględnienia tak zwanej grupy kontrolnej pozornej (poddanej iniekcji PBS), ze względu na dużą zamieralność zarodków. W doświadczeniu wykorzystano jedno stężenie nanocząstek – 500 μg/ml ze względu na najefektywniejsze oddziaływanie w trakcie przeprowadzonych uprzednio badań pilotażowych. Procedura postępowania z jajami, techniką iniekcji oraz kontrola w trakcie inkubacji były tożsame z opisanymi w panelu I. Przeżywalność zarodków była wyznaczona stosunkiem liczby żywych zarodków w 20. dobie inkubacji do liczby jaj zapłodnionych w grupie. W 20. dniu inkubacji część zarodków wyjęto z jaja, zważono i uśmiercono. Pobrana w trakcie dekapitacji krew na skrzep (10 prób na grupę) została zabezpieczona do późniejszych analiz zgodnie z podrozdziałem 3.2.2. Z każdej grupy losowo wybrano 10 zarodków, od których pobrano wątrobę, mięśnie piersiowe oraz kości – udową i piszczelową. W surowicy oznaczono podstawowe parametry biochemiczne, wymienione w doświadczeniu I. Wykorzystano tę samą metodę do oznaczania koncentracji MDA i GSH, jak w doświadczeniu I.

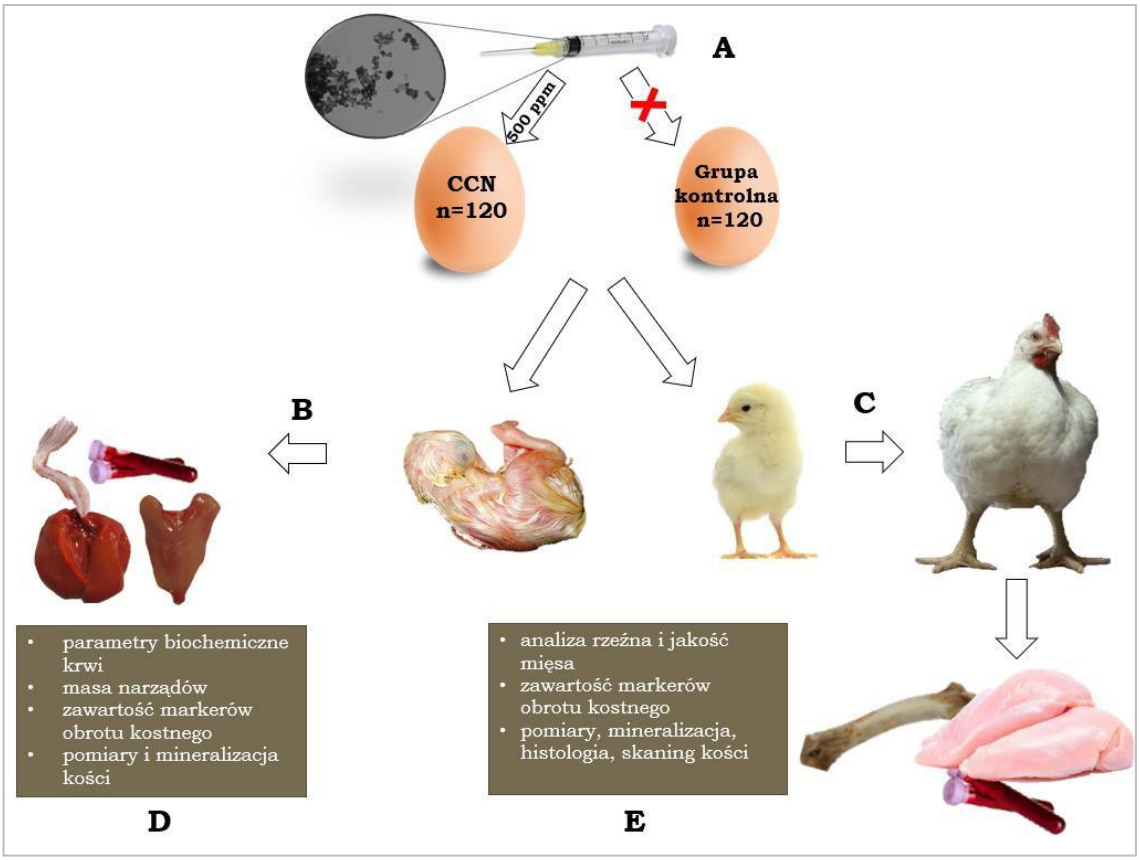
Oczyszczone kości zarodków (udowa i piszczelowa) zostały zmierzone (długość i średnica na środku trzonu) z użyciem suwmiarki elektronicznej oraz zważone na wadze analitycznej. Następnie przeprowadzono kontrolę siły łamania na maszynie wytrzymałościowej Zwick. Kolejno wykonano szereg analiz na sześciu prawych kościach udowych i piszczelowych z każdej grupy tzn. oznaczenie Ca i P przy użyciu spektrometru emisji atomowej ICP-AES Thermo iCAP 6500 DUO, przygotowano homogenaty z kości lewej kończyny (na PBS), w których określono zawartość białka (BCA, Merck, Warszawa, Polska), a następnie oznaczono OC testem ELISA (Immunogen, Warszawa, Polska). OC oraz BALP zostały także oznaczone w surowicy krwi zarodków. Odczyt przeprowadzono na analizatorze płytkowym Tecan's NanoQuant Infinite M200 PRO.

Część jaj w doświadczeniu została poddana pełnemu okresowi inkubacji - do 21. dnia. Po wykluciu, jednodniowe pisklęta zostały przeznaczone do standardowego odchowu. W grupie znajdowało się 90 sztuk piskląt. W trakcie

odchowu kontrolowano masę ciała przy zmianie mieszanek (podrozdział 3.2.3.), a na koniec odchowu wyliczono podstawowe wskaźniki produkcyjne kurcząt. Cały cykl trwał 42 dni. W trakcie dekapitacji pobrano krew od sześciu kogutów o średniej masie ciała dla danej grupy. Ptaki następnie pozbawiono upierzenia iwypatroszono. Po 24 godzinnym schłodzeniu tuszek, przeprowadzono dysekcję i analizę rzeźną (podrozdział 3.2.3). Pobrane w trakcie dysekcji mięśnie piersiowe poddano homogenizacji i przeznaczono do następujących analiz: pH zmierzone od 24h od uboju wykorzystując pehametr CP-411 z elektrodą szklaną (Elmetron, Zabrze, Polska), określono parametry barwy przy użyciu kolorymetru CR-410 (Minolta Ca. Ltd., Osaka, Japonia), gdzie każdy pomiar powtarzano dwukrotnie. Uzyskane wartości reprezentowały skalę jasności (parametr L) oraz stopień wysycenia barwą czerwoną/zieloną (parametr a) i żółtą/niebieską (parametr b).

Podczas dysekcji tuszek, z każdej pobrano prawą i lewą kość udową do analiz. Kości zmierzono i zważono, by kolejno lewe kości przeznaczyć do określenia siły łamania (podrozdział 3.2.3). Następnie pobrano 3 g tkanki (z części przy nasadzie bliższej), które zmiażdżono w moździerzu. Rozdrobnione kości spalono, a następnie określono w nich zawartość Ca, P, Mg, Mn, Zn i Cu przy użyciu spektrometru emisji atomowej ICP-AES Thermo iCAP 6500 DUO (Rutkowska, 1981). Z kości udowych pobrano fragment z okolicy nasady bliższej o masie ok. 0,2 g, rozdrobniono i liofilizowano przez 24 h. Następnie z kości ekstrahowano białko w 1 ml buforu RIPA (Merck, Warszawa, Polska) przez 5 dni, po czym homogenizowano. W homogenatach oznaczono zawartość białka oraz koncentrację OC z wykorzystaniem testu ELISA. Prawe kości zostały poddane skanowaniu komputerowemu z wykorzystaniem wielorzędowego tomografu CT (750 Revolution CT, Waukesha, Wisconsin, Stany Zjednoczone) zgodnie z protokołem Gemstone Spectral Imaging (GSI). Obrazy analizowano w programie AW VolumeShare7 (GE Healthare, Waukesha, Wisconsin, Stany Zjednoczone).Narzędzie do automatycznego pomiaru zostało użyte aby dopasować okno pomiarowe do rozmiaru kości w trzech płaszczyznach – strzałkowej, czołowej i osiowej. Po ustawieniu odpowiedniego progu, określono średnią względną gęstośćwyrażoną w jednostkach Hounsfielda (HU) dla każdej kości oraz objętość kości (cm^3). Ponadto, wyznaczono średnią objętość kości dla wystandaryzowanych wartości gęstości – 500 i 1000 HU.

Z prawych kości udowych wycięto ok. 0,5 cm długości skrawki, które natychmiast utrwalono w 10% roztworze formaliny na okres 72 h. Następnie skrawki dekalcyfikowano w 15% buforze EDTA (pH = 7.4) (Merck, Warszawa, Polska) przez 1 miesiąc. Dekalcyfikowane kości zostały poddane rosnącemu stężeniu etanolu (5-100%) celem odwodnienia, kolejno odtłuszczone w ksylenie i zatopione w parafinie. Skrawki o grubości 6 μm przeznaczone do barwienia histologicznego zostały przygotowane na mikrotomie Leica RM 2265 (Leica Biosysytems, Nussloch, Niemcy). Wykonano barwienie podstawowe hematoksyliną i eozyną oraz barwienie alizaryną red S (Merck, Warszawa, Polska). Barwienie alizaryną przeprowadzono w następujący sposób: odtłuszczone i ponownie nawodnione skrawki parafinowe były zanurzone w 2% roztworze barwnika przez 2 minuty. Następnie zanurzono je w acetonie, a potem w mieszaninie acetonu i ksylenu (50/50) przez 2 minuty (zmodyfikowana metodyka Guo i wsp., 2016a). Wizualizację obrazów przeprowadzono na mikroskopie świetlnym Nikon Eclipse 90i wyposażonym w kamerę Nikon Digital DS.-U1 (Nikon Corporation, Tokio, Japonia). Wizualizacje przeprowadzano dla 18 preparatów z każdej grupy. Stopień mineralizacji (intensywność barwy czerwonej) zmierzono przy użyciu programu NIS-Elements „D". Schematyczny układ doświadczenia 2 przedstawiono na rys. 2.



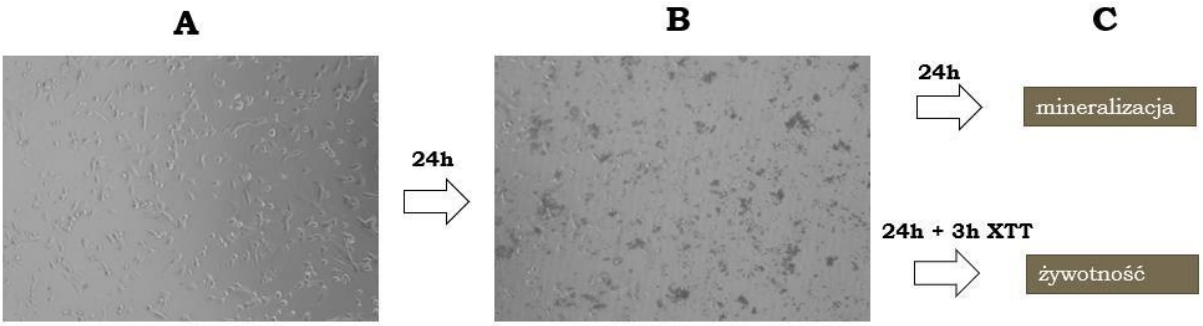
Rys. 2. Schemat doświadczenia 2. Objaśnienie skrótów: A – iniekcja 200 µl hydrokoloidu CCN w stężeniu 500 ppm do białka jaja; B – pobranie zarodków w 20. dobie inkubacji, izolacja wątroby, mięśni piersiowych, krwi, kości kończyny dolnej; C – losowy wybór 90 piskląt przeznaczonych do dalszego odchowu, pobór krwi, dysekcja, analiza rzeźna, izolacja kości i mięśni piersiowych od kurcząt brojlerów po zakończonym odchowie; D – wykonywane analizy na pobranym materiale od zarodków; E – wykonywane analizy na pobranym materiale od kurcząt.

Panel dodatkowy. Ocena żywotności i stopnia mineralizacji ptasich komórek osteogennych po ekspozycji na rosnące stężenie CCN (Matuszewski i wsp., 2021).

Celem doświadczenia była ocena żywotności i stopnia mineralizacji ptasich komórek osteogennych po ekspozycji na rosnące stężenia CCN. Żywotność określono wykorzystując test kolometryczny do pomiaru stopnia proliferacji komórek – XTT (Life Technologies, Taastrup, Dania). Komórki zostały umieszczone na płytkach 96-dołkowych (5×10^3 komórek na dołek) i inkubowane przez 24h (37°C, 5% CO_2). Następnie usunięto 50 µl pożywki, a do dołków dodano 50 µl różnych stężeń nanocząstek CCN – 5, 10, 25, 50 i 100 µg/ml i inkubowano przez

koleje 24h. W dalszej kolejności dodano odczynnik XTT w ilości 50 µl i inkubowano dodatkowo przez 3h. Gęstość optyczną przy długości fali 450 nm odczytano na analizatorze mikropłytek Infinite M200 (Tecan, Durham, Karolina Północna, Stany Zjednoczone). Żywotność określono procentowo stosując następujący wzór: $OD_{próba} - OD_{próba\ ślepa})/(OD_{próba\ kontrolna} - OD_{próba\ ślepa})$ x 100, gdzie $OD_{próba}$ oznacza gęstość optyczną komórek poddanych działaniu nanocząstek, $OD_{próba\ kontrolna}$ gęstość optyczną dla próby kontrolnej, a $OD_{próba\ ślepa}$ gęstość optyczną dla dołka bez komórek (z pożywką hodowlaną).

W celu określenia stopnia mineralizacji, komórki naniesiono na płytkę 6-dołkową (1 x 10^5 komórek na dołek) i inkubowano przez 24h. Po upływie doby do hodowli dodano rosnące stężenia nanocząstek – 5, 10, 20, 50 i 100 µg/ml poddając je dobowej inkubacji. Do grupy kontrolnej nie dodawano żadnego stężenia nanocząstek. Po 24h komórki zostały utrwalone w 4% roztworze paraformaldehydu i barwione 2% alizaryną (Merck, Warszawa, Polska) (Jeon i wsp., 2018). Wizualizacje prowadzono na mikroskopie optycznym (TL-LED, Leica Microsystems, Wetzlar, Niemcy). Schematyczny układ doświadczenia przedstawiono na rys. 3.



Rys. 3. Schemat doświadczenia *in vitro.* Objaśnienie skrótów: A – osteogenne komórki ptasie wyizolowane z kości udowej zarodka kury; B – komórki po 24 inkubacji traktowane rosnącymi stężeniami nanocząstek CCN; C – wykonywane analizy po 24h inkubacji z nanocząstkami w różnych stężeniach.

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3.4. Techniki badawcze

- Analiza potencjału Zeta nanocząstek hydroksyapatytu i węglanu wapnia z użyciem Zetasizer Nano-ZS90 (Malvern, Malvern, Worcestershire, Wielka Brytania).
- Wizualizacja nanocząstek związków wapnia z zastosowaniem transmisyjnego mikroskopu elektronowego Morgagni 268D z kamerą szerokokątną Olympus Morada (FEI, Oregon, Stany Zjednoczone).
- Analiza spektrofotometryczna markerów obrotu kostnego – OC, OPG, PINP, BALP, markerów oksydacji (GSH, MDA, GSH-Px, GR, SOD, TAS) w tkankach i narządach, oznaczenia poziomu białka z użyciem Tecan's NanoQuant Infinite M200 PRO (Tecan, Grödig, Austria) i analiza żywotności komórek po ekspozycji na nanocząstki z użyciem spektrofotometru płytkowego Infinite M200 (Tecan, Durham, Karolina Północna, Stany Zjednoczone).
- Wizualizacja kości z wykorzystaniem i skaningowego mikroskopu elektronowego (FEI, Hillsboro, Stany Zjednoczone).
- Przygotowanie skrawków o grubości 6 μm przeznaczonych do barwienia histologicznego przy użyciu mikrotomu Leica RM 2265 (Leica Biosysytems, Nussloch, Niemcy).
- Wizualizacja preparatów z kości barwionych H&E i alizaryną na mikroskopie świetlnym Nikon Eclipse 90i wyposażonym w kamerę Nikon Digital DS.-U1 (Nikon Corporation, Tokio, Japonia). Stopień mineralizacji zmierzono przy użyciu programu NIS-Elements „D".
- Analiza siły łamania kości zarodków kury i kurcząt brojlerów z wykorzystaniem maszyny wytrzymałościowej Zwick Z 0.5, Zwicki-Line, Ulm, Niemcy).
- Określenie zawartości Ca i P w kościach zarodków kury oraz zawartość Ca, P + mikroelementów w kości udowej kurcząt brojlerów (Mg, Mn, Zn i Cu) przy użyciu spektrometru emisji atomowej ICP-AES Thermo iCAP 6500 DUO (Thermo Fisher Scientific, Waltham, Massachusetts, Stany Zjednoczone).
- Skanowanie (tomografia) kości w celu określenia gęstości i objętości kości z wykorzystaniem wielorzędowego tomografu CT (750 Revolution CT, Waukesha, Wisconsin, Stany Zjednoczone) zgodnie z protokołem Gemstone

Spectral Imaging (GSI). Analiza obrazu w programie AW VolumeShare7 (GE Healthare, Waukesha, Wisconsin, Stany Zjednoczone).

- Ocena wybranych wyróżników jakości mięśnia piersiowego tzn. pH wykorzystując pehametr CP-411 z elektrodą szklaną (Elmetron, Zabrze, Polska), i parametrów barwy przy użyciu kolorymetru CR-410 (Minolta Ca. Ltd., Osaka, Japonia),
- Wizualizacja stopnia mineralizacji komórek po ekspozycji na nanocząstki związków wapnia z użyciem mikroskopu optycznego (TL-LED, Leica Microsystems, Wetzlar, Niemcy).

3.5. Analiza statystyczna

Wyniki analizowano w układzie analizy wariancji jednoczynnikowej – ANOVA. Istotności między grupami były określane przy użyciu testu Tukey'a. Analizę statystyczną prowadzono w programie IBM SPSS Statistics, wersja 21.0. Różnice o $p \leq 0,05$ uznawano za istotne statystycznie.

4. Omówienie głównych wyników prac eksperymentalnych.

4.1. Ocena wpływu różnych stężeń HA-NP aplikowanych *in ovo* do białka na rozwój zarodka kury, ze szczególnym uwzględnieniem rozwoju układu kostnego (Matuszewski i wsp., 2020b)

Na podstawie uzyskanych wyników stwierdzono, że aplikacja *in ovo* HA-NP. w zakresie stężeń od 50 do 500 µg/ml nie wpłynęła negatywnie na przeżywalność zarodków kury w 20. dobie inkubacji. Zarodki wykazywały prawidłowy wzrost i rozwój, co generalnie zostało potwierdzone także w badaniu Ahmadzadeh i wsp. (2019). Nie wykazano także różnic w masie ciała i masie mięśni piersiowych, zaobserwowano zaś istotne zmniejszenie masy wątroby w grupie po iniekcji 100 µg/ml hydrokoloidu HA-NP. Wykazano, że masa kości udowej i piszczelowej w grupie 500 HA-NP, została istotnie zredukowana, pomimo relatywnie najwyższej średnicy kości udowej w tej grupie. Taka sytuacja może być związana z toksycznym oddziaływaniem HA-NP na zarodek kury. Efekt toksyczny będzie zależeć od rodzaju

nanocząstek i wynikających z niego specyficznych właściwości (struktura, morfologia) (Matuszewski i wsp., 2020b; Ajita i wsp. 2015). Pozostałe wartości pomiarów morfometrycznych nie wykazywały znaczących różnic. Podobnie nie zaobserwowano różnic w sile łamania kości pomiędzy grupami. Wizualizacje kości z mikroskopu elektronowego pokazały ich prawidłową budowę mikroarchitektoniczną u wszystkich badanych grup. Mineralizacja obydwóch rodzajów kości wyrażona w zawartości Ca i P również nie była statystycznie istotna pomiędzy grupami. Można jednak zaobserwować pewną tendencję mówiącą o przewadze zawartości Ca i popiołu surowego w kościach w grupie 500 HA-NP. Nie stwierdzono różnic dla parametrów biochemicznych krwi, co sugeruje prawidłowe funkcjonowanie wątroby i nerek. W przypadku markerów stresu oksydacyjnego zaobserwowano różnicę statystycznie istotną pomiędzy grupą kontrolą, a doświadczalnymi w koncentracji GSH, gdzie najniższe jego stężenie było w grupie kontrolnej, a istotnie wyższe w trzech grupach doświadczalnych ($p \leq 0.05$). Natomiast najwyższe stężenie MDA wystąpiło w grupie kontrolnej, a najniższe w grupie 500 HA-NP ($p \leq 0.05$). Iniekcja HA-NP do jaja spowodowała u zarodków zwiększenie ochrony antyoksydacyjnej, ponieważ wzrastające stężenie GSH jest skorelowane z wyższą koniecznością neutralizacji reaktywnych form tlenu (Wu i wsp., 2004). Dla markerów stresu oksydacyjnego wykazano, że najwyższą koncentracją SOD w mięśniu piersiowym odznaczała się grupa kontrolna, zaś w wątrobie – grupa 500 HA-NP. Całkowity status oksydacyjny mierzony w mięśniu piersiowym był istotnie wyższy w grupie 500 HA-NP, gdzie analogicznych różnic w wątrobie nie zaobserwowano. Generalnie można zauważyć, że wartości SOD i TAS były w wyższe w tkance jaką jest mięsień piersiowy. Należy jednak podkreślić, że dane dotyczące regulacji antyoksydacyjnej w ptasim zarodku są ograniczone, dlatego potrzeba kolejnych badań w tym aspekcie wydaje się być zasadna. Zawartość wybranych markerów obrotu kostnego mierzonych w surowicy – OPG i BALP nie różniła się istotnie po wprowadzeniu czynnika jakim była iniekcja *in ovo* do białka HA-NP. Zaobserwowano jednak różnicę w koncentracji PINP, gdzie jego zawartość była wyraźnie wyższa w grupie 100 HA-NP, dla OC najwyższe stężenie odnotowano w grupie 500 HA-NP ($p \leq 0.05$). Dla kości udowej i mierzonej w niej markerach obrotu kostnego – PINP i OC, zaobserwowano różnice statystycznie istotne ($p \leq 0.05$) dla OC, której zawartość była najwyższa dla 500 HA-NP i

charakteryzowała się ona wzrostem wprost proporcjonalnym do wzrostu stężenia użytych HA-NP. Koncentracja OC w tkankach wydaje się być najbardziej podlegającą wpływowi zewnętrznego czynnika jakim są HA-NP. W sytuacji gdy stężenie wolnej OC w surowicy rośnie, może to sugerować zmniejszenie stopnia mineralizacji (na skutek zwiększonego obrotu kostnego), co jest efektem niekorzystnym. Jednak jej zwiększona zawartość w kościach mówi o lepszej mineralizacji. W tym przypadku, zwiększenie koncentracji OC w surowicy i kościach może być spowodowane blokowaniem procesu mineralizacji w odpowiedzi na zewnętrzne źródło hydroksyapatytu, jednak wymagane jest przeprowadzenie dodatkowych badań celem potwierdzenia tej hipotezy.

4.2. Ocena wpływu wybranego stężenia CCN podanego *in ovo* do białka na rozwój zarodka kury a także na wyniki produkcyjne, jakość mięsa i kości kurcząt brojlerów w 42. dniu życia (Matuszewski i wsp., 2021)

Wyniki przeprowadzonego doświadczenia wykazały, że CCN podane drogą iniekcji bezpośredniej *in ovo* do białka w stężeniu 500 μg/ml nie oddziałują w sposób negatywny na rozwój zarodka kury i przeżywalność – ta była na poziomie powyżej 90% w obu grupach w doświadczeniu. Podobny rezultat osiągnięto w doświadczeniu Salary i wsp. (2017), gdzie przeżywalność zarodków była na podobnym poziomie po iniekcji *in ovo* do białka CCN. Aczkolwiek przeżywalność zarodków w tym badaniu była generalnie na niższym poziomie, pomimo zastosowania niższego stężenia CCN. Przeżywalność zarodków zależy od rodzaju aplikowanego nanomateriału, miejsca iniekcji, jak również czasu iniekcji (Matuszewski i wsp. 2020b) oraz samej jakości jaj wylęgowych (Nasri i wsp., 2020). Masa ciała zarodka oraz masa mięśni piersiowych nie różniły się pomiędzy grupami. Natomiast u zarodków z grupy poddanej iniekcji (CCN) stwierdzono wyższą masę wątroby ($p \leq 0.05$), co może sugerować wpływ CCN na różnicowanie się rozwoju narządów w okresie embrionalnym. Parametry biochemiczne krwi, za wyjątkiem glukozy, której koncentracja była niższa w grupie CCN ($p \leq 0.05$), nie różniły się istotnie u badanych grup. Zaobserwowano również, że stężenie MDA było znacząco niższe w grupie CCN ($p \leq 0.05$). Istotnie wyższa koncentracja markerów jak np. ALT

czy AST może wskazywać na dysfunkcje metaboliczne (Guo i wsp., 2016b), co jednak nie zostało stwierdzone w badaniu własnym. Malejące stężenie ALP może z kolei nasuwać fakt wystąpienia problemu związanego z metabolizmem kości. W badaniu własnym także nie wykazano istotnych różnic w tym parametrze jednak w doświadczeniu Salary i wsp. (2017) wyższe stężenie ALP wpłynęło się na wyższą mineralizację kości. Analiza pomiarów kości wykazała, że masa kości piszczelowej była wyższa w grupie CCN, co więcej w obu rodzajach zaobserwowano wyższą zawartość Ca i P w grupie CCN ($p \leq 0.05$), co sugeruje lepszą mineralizację tej tkanki u zarodków.

Po trwającym 42 dni odchowie kurcząt brojlerów wykazano, że iniekcja *in ovo* do białka CCN w stężeniu 500 µg/ml nie wpłynęła negatywnie na parametry produkcyjne kurcząt począwszy od pierwszego dnia życia do ostatniego. Masa początkowa piskląt była na podobnym poziomie, nie stwierdzono istotnych różnic podczas ważeń kontrolnych w późniejszych okresach odchowu. FCR także nie różnił się istotnie pomiędzy grupami (1,59 w CCN vs. 1,51 w grupie kontrolnej), a śmiertelność w trakcie całego odchowu w obydwóch grupach była na niskim, akceptowalnym poziomie (1,54% w CCN vs. 2,49 w grupie kontrolnej). Wyniki te są zgodne z wynikami Salary i wsp. (2017), którzy również nie dowiedli wpływu CCN podanego *in ovo* na parametry produkcyjne kurcząt brojlerów. Wyniki analizy rzeźnej również wskazywały na to, że kurczęta rozwijały się prawidłowo i nie wykazano różnic w wydajności rzeźnej (ok. 78% dla obu grup), procentowego udziału mięśni piersiowych, mięśni nóg i podrobów. Wyróżniki jakości mięśni piersiowych to jest pH i barwa różniły się pomiędzy grupami jedynie w odniesieniu do parametru barwy a*, na co wpływ mógł mieć sam proces uboju.

W pobranych w trakcie dysekcji tuszek kości udowych, stwierdzono wyższą zawartość Ca w grupie CCN ($p \leq 0.05$), natomiast zawartość P, czy mikroskładników takich jak Mg, Mn, Zn i Cu nie różniła się pomiędzy grupami. Podobny rezultat wykazali Salary i wsp. (2017), otrzymując różnice w zawartości Ca i P w kościach kurcząt brojlerów po aplikacji *in ovo* CCN, nawet w mniejszym stężeniu. Wykazano silną tendencję ($p = 0.053$) w relatywnej gęstości mineralnej w grupie CCN w porównaniu do grupy kontrolnej, co dodatkowo zostało potwierdzone zwiększoną zawartością Ca. Jest to istotne ze względu, że 99% Ca znajdującego się w organizmie

pochodzi z kości, gdzie bierze udział w formowaniu hydroksyapatytu (wraz z P). Opierając się na uzyskanych wynikach można stwierdzić, że iniekcja *in ovo* do białka CCN wywołuje dalekosiężne efekty polepszenia kalcyfikacji kości kurcząt. Masa kości, ich długość oraz wartość siły łamania były na podobnym poziomie. Na wizualizacjach preparatów histologicznych barwionych alizaryną można było zauważyć niższą intensywność barwy czerwonej w grupie kontrolnej w stosunku do grupy CCN (wartości histogramu 200.9 dla grupy CCN vs. 196.0 dla grupy kontrolnej; $p \leq 0.05$). Mniejsza intensywność barwy części zbitej i gąbczastej kości udowej potwierdziła słabszą mineralizację kości w grupie kontrolnej (słabsza kalcyfikacja). Zwiększona zawartość Ca w kościach może wpływać na wyższą odporność na złamania, jednak samo badanie siły łamania tego nie potwierdziło. Wytrzymałe kości udowe, ze względu na pełnione funkcje nośne masy ciała ptaka, są o tyle istotne, ponieważ ograniczają występowanie deformacji układu szkieletowego (Mroczek-Sosnowska i wsp., 2017). Wyniki dotyczące koncentracji OC w surowicy i kościach udowych zarodków oraz kurcząt brojlerów wykazały, że jej wyższa zawartość wystąpiła w kości udowej zarodków z grupy CCN, gdzie jednocześnie wyższa zawartość tego markeru w surowicy zarodków była stwierdzona w grupie kontrolnej, co zostało potwierdzone statystycznie ($p \leq 0.05$). Świadczy to o zwiększonym procesie obrotu kostnego w kościach zarodków (podwyższenie koncentracji wolnej OC we krwi). U dużych ptaków z kolei poziom OC w surowicy określono na zbliżony, jednak jej stężenie w kości udowej było istotnie wyższe w grupie CCN. Wynik ten również bezpośrednio przekłada się na wyższy stopień mineralizacji w grupie doświadczalnej ($p \leq 0.05$). Można zatem stwierdzić, że CCN podane *in ovo* do białka mogą efektywnie modulować procesy obrotu kostnego u zarodka kury, a w późniejszym okresie u kurcząt brojlerów, poprzez regulację OC – białka odpowiedzialnego za wiązanie hydroksyapatytu.

Ocena żywotności i stopnia mineralizacji ptasich komórek osteogennych po ekspozycji na rosnące stężenie CCN (Matuszewski i wsp., 2021).

Wyniki testu cytotoksyczności (XTT) CCN w zakresie stężeń od 5 do 100 µg/ml na ptasie komórki osteogenne wykazały, że CCN w żadnym z wybranych

stężeń nie wpływają negatywnie na żywotność komórek (brak dawkozależnego efektu toksycznego). Co więcej, żywotność komórek wzrastała wprost proporcjonalnie do rosnącego stężenia CCN, sugerując tym samym stymulujące oddziaływanie tych nanocząstek na żywotność komórek na skutek ich silnych właściwości osteokondukcyjnych. Test na mineralizację komórek wykazał znacznie intensywniejsze wybarwienie zwapnionych obszarów w obrębie komórek przy zastosowaniu wyższych stężeń nanocząstek. Oznacza to zatem efektywniejszą kalcyfikację w hodowli poddanej ekspozycji na wyższe stężenie CCN.

Jednocześnie przeprowadzono także ocenę przeżywalności i stopnia mineralizacji ptasich komórek osteogennych po ekspozycji na rosnące stężenie HA-NP, aczkolwiek wyniki te nie zostały opublikowane.

5. Podsumowanie

Podsumowując należy stwierdzić, iż HA-NP podane *in ovo* do białka w zakresie stężeń 50-500 μg/ml nie wpływają negatywnie na przeżywalność zarodków, ich masę ciała i masę narządów. Dodatkowo HA-NP wpływają na modulację aktywności wybranych markerów stresu oksydacyjnego. Wysokie stężenie GSH w surowicy zarodków w grupie po iniekcji różnymi stężeniami HA-NP jest prawdopodobnie skorelowane z potrzebą detoksykacji ROS, zatem stężenia 50-500 μg/ml mogą wywołać efekt toksyczny dla zarodka. HA-NP wpływają również na modulację koncentracji markerów obrotu kostnego, szczególnie OC która osiągnęła najwyższe stężenie w grupie 500 μg/ml w kościach, jak i w surowicy. Sugerować to może blokowanie procesu mineralizacji na skutek zewnętrznego czynnika w postaci HA-NP, co odzwierciedlają wyniki pomiarów morfometrycznych kości – najniższa masa kości w grupie 500 HA-NP.

Przeprowadzone badania wykazały, że CCN są materiałem biokompatybilnym, o właściwościach osteokondukcyjnych. Iniekcja bezpośrednia do białka jaja CCN w stężeniu 500 μg/ml nie jest szkodliwa dla zarodka kury i nie wpływa na przeżywalność. CCN nie wywołują negatywnych efektów w koncentracji wybranych parametrów biochemicznych krwi. Dodatkowo, wpływają na zwiększenie Ca i P w kościach nóg u zarodków. Podane *in ovo* do białka CCN wywołują szereg zmian ogólnoustrojowych, które wpływają na efekt dalekosiężny

– podwyższenie zawartości OC w kościach kurcząt, a tym samym poprawę ich jakości na skutek lepszej mineralizacji. Potwierdzeniem biokompatybilności i osteokonduktywności CCN są również wyniki badań na mniej złożonym modelu badawczym – ptasich komórkach osteogennych, w których wykazano wyższą żywotność i mineralizację przy ekspozycji na wyższe stężenia CCN.

Wyniki przeprowadzonych doświadczeń umożliwiły weryfikację założeń pracy, jednocześnie stanowią bazę do dalszych badań nad wykorzystaniem nanocząstek związków wapnia w odżywianiu funkcjonalnym *in ovo* jak również w żywieniu tradycyjnym, jako alternatywnych źródeł wapnia.

6. Wnioski

1. HA-NP podane *in ovo* do białka w stężeniach 50-500 µg/ml nie wpływają negatywnie na rozwój zarodka kury, jego masę, masę narządów oraz przeżywalność zarodków w 20. dniu inkubacji.

2. HA-NP podane *in ovo* do białka w stężeniu 50-500 µg/ml wykazują działanie modulujące status redoks zarodka kury, odzwierciedlony w podwyższonym stężeniu GSH w surowicy zarodka kury, jako odpowiedź na toksyczne działanie HA-NP.

3. HA-NP podane *in ovo* do białka wykazują działanie modulujące procesy obrotu kostnego zarodka kury. Wraz ze wzrostem stężenia HA-NP zwiększa się koncentracja OC w surowicy i kościach. Obniżona masa kości udowej i piszczelowej w grupie otrzymującej najwyższe stężenie HA-NP jest efektem niekorzystnym.

4. CCN podane *in ovo* do białka w stężeniu 500 µg/ml nie wpływają negatywnie na rozwój zarodka, jego masę, masę narządów i przeżywalność zarodków w 20. dniu inkubacji. Wykazują działanie modulujące procesy obrotu kostnego zarodka kury, podwyższając stężenie OC w kości udowej i obniżając stężenie OC w surowicy, przekładające się na wyższy poziom mineralizacji kości.

5. CCN podane *in ovo* do białka w stężeniu 500 µg/ml nie wpływają negatywnie na parametry produkcyjne kurcząt brojlerów określane po 42. dniu odchowu, nie wypływają na wyniki analizy rzeźnej, pH i barwę mięśnia piersiowego.

Wywołują one efekt w postaci poprawy kalcyfikacji kości udowej, popartej wyższą koncentracją OC w kości, a niższą w surowicy oraz intensywnością wybarwienia złogów wapnia.

6. CCN w zakresie stężeń 5-100 μg/ml wpływają stymulująco na żywotność ptasich komórek osteogennych oraz na ich mineralizację *in vitro* wprost proporcjonalnie do wzrostu stężenia nanocząstek.

Uogólnienia:

Nanocząstki związków wapnia – HA-NP i CCN ze względu na różniące je właściwości fizykochemiczne odznaczają się inną interakcją i biokompatybilnością z modelem badawczym jakim jest zarodek kury. Z tego względu w różny sposób wpływają na biologiczną reakcję zarodka.

Ze względu na korzystniejszy wpływ CCN aniżeli HA-NP podanych na rozwój układu kostnego i jego metabolizm oraz mniejszą toksyczność wydają się być bardziej rekomendowane do zastosowania w odżywianiu funkcjonalnym *in ovo* mającym na celu poprawę jakości kości.

Zastosowanie CCN w technologii *in ovo* stwarza możliwość potencjalnego wykorzystania tego materiału w produkcji drobiarskiej. Jednakże, wciąż istnieje potrzeba przeprowadzenia kolejnych badań w tym zakresie, ponieważ zastosowanie nanocząstek związków wapnia w chowie i hodowli drobiu to temat wciąż słabo poznany.

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Calcium and phosphorus and their nanoparticle forms in poultry nutrition

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SUMMARY

The modern broiler chicken industry is connected with various disorders of the skeletal system. The fast-growing birds by increased weight gain often have leg problem which leads to economic losses. The correct bone development of broilers is highly correlated with calcium and phosphorus ratio (about 2:1) so providing these macroelements with diet seemed to be fundamental. The most common inorganic sources such as limestone, mono- and di-calcium phosphates are commonly used nowadays. The doses in feed generally are in the range of 6–6.5 g/kg for Ca and 2–3.5 g/kg for P, depending on the supplementation of phytase. However, the bioavailability of inorganic sources is poorer than organic sources. This fact is important in reference to their impact on the environment. Because of the continuous search for alternative sources of calcium or phosphorus, with better bioavailability due to, for example, their size, the scientific area of nanotechnology arouses increasing interest. It is well-known nanoparticles have a great potential even at very low doses. Some research focused on calcium-phosphorus compounds already demonstrated no negative effect on birds' health, improvement in production results and bone quality, opportunity to use lower dosages of nano sources and decreasing content of Ca and P in excreta (by *ca*. 50%). Thus, this aspect may be the new trend during the next years. However, the further studies should be performed.

KEYWORDS

Broiler; nutrition; calcium; phosphorus; nanoparticles; bone quality

Introduction

Intensive selection for body weight gain performed in flocks of broiler chickens has led to its increase by almost 300% within the last 50 years (Knowles *et al*. 2008; Petracci and Cavani 2012). The fast-growing bird is unable to develop a completely mature skeleton that would manage to carry such a heavy body. Contemporary broiler chickens can spend most of the time in lying position (from 76% to 86% of time within a day), which increases with age (Morris 1993). The heavy load imposed on bones of the limbs leads to multiple pathologies, including deformations, osteoporosis and various infections (Thorp and Waddington 1997; Rath *et al*. 1999; Fleming 2008). Economic losses caused by various disorders of the skeletal system can reach 120 million USD in the United States annually (Cook 2000). In the Great Britain, not less than 27% of the birds are estimated to

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suffer from locomotor system disorders (Knowles *et al.* 2008). These numbers indicate these disorders pose a serious problem in the production of broiler chickens.

Bone damage may directly influence meat quality, e.g. they may cause muscle effusions which are highly undesirable in the final products (Gregory and Wilkins 1990). There are two main types of bone deformation, tibial dyschondroplasia (TB) and rickets (Hulan *et al.* 1985). Manifestations of dyschondroplasia include degenerations of cartilage in the head of the tibia in chickens aged three-eight weeks, most often seen in the heaviest birds (Edwards 1984). Pathologies in the morphology and functions of tibial cartilage were first described by Leach and Nesheim (1965). Several years later, Edwards (1984) reported that TB affects birds fed diets with an inappropriate calcium to phosphorus ratio, in particular those having a low calcium and high phosphorus. Typical traits of this disease include an unusual shape as well as white and dull cartilage. Histological examinations point to cartilage retardation, involving its demineralisation (decalcification in particular) and to the atrophy of blood vessels (Farquharson and Jefferies 2000; Webster *et al.* 2003). Rickets are triggered by the imbalance between calcium and phosphorus as well as vitamin D_3 in the diet, which, in growing birds, is manifested by irregularities in calcification and, generally, in the mineralisation of long bone cartilage (Jubb *et al.* 2007; Samuelson 2007). There are two main types of rickets in broiler chickens. The first one is hypophosphatemic rickets induced by low phosphorus absorption from diet. The histological image of epiphyseal cartilage reveals agglomeration of the layer of proliferating cells coupled with metaphyseal vascularisation. The second is hypocalcaemia rickets, triggered by calcium deficiency. The histological image differs in this case, revealing noticeable agglomeration of the proliferating layer of the epiphyseal cartilage (Lacey and Huffer 1982).

In the case of broiler chickens, deformities of their locomotor system depend on the strength of bones, in particular the femur (*os femoris*), tibia (*tibia*), fibula (*fibula*), and pad skeleton (*skeleton pedis*), considering their supporting and weight carrying functions. Bone strength is determined by multiple factors, including bird growth rate, bird age, bird genotype, feeding system, sex, infectious factors, as well as endocrinal metabolism, toxins in feedstuffs and bird handling, e.g. mechanical damages caused during catch and slaughter (Rath *et al.* 2000).

Considering the nutritional factors, it is reasonable to pay attention to the two major macroelements involved, the intake and common ratio of Ca and P which are necessary to ensure bone strength. These elements are the main contributors to bone mineral structure, where they occur in the form of hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) (Scott *et al.* 1982; Turek 1984). The mineralisation of bones affects their strength which, in turn, is determined by the mass, volume or microarchitectural organisation and the degree of mineralisation of the bone matrix (Boivin and Meunier 2002). Conventional indicators of the bone mineral status include various measurements and tests, such as breaking strength (Park *et al.* 2003; Kim *et al.* 2006), mineral density (Kim *et al.* 2006), crude ash content (Garlich *et al.* 1984; Park *et al.* 2003) and elemental mineral content, including Ca and Pas well as Mg, Cu and Mn (Akpe *et al.* 1987; Kim *et al.* 2006).

Nanoparticles are defined as having sizes smaller than 100 nm but larger than 0.2 nm, that are characterised by varied physical, chemical and biological properties as a result of the ratio of area to volume compared to their larger counterparts (e.g. ionic scale; Roco 1999). Nanobiotechnology affords new possibilities in many fields of science like chemistry, physics, biology or medicine, as well as in animal science. The research in this sector has

shown that various nanoparticles could be used as an alternative to antibiotics (Pineda *et al.* 2012), growth promoters (Andi *et al* 2011) or as a better opportunity for certain feed additives (Hassan *et al* 2013; Ibrahim*et al* 2017). It has been hypothesised that different Ca nanoparticles, because of their high physical reactivity, could be an alternative to conventional forms as they can be supplied in much smaller doses in chicken diets. This would have the added advantage of significantly reducing excretion of these minerals into the environment.

Chicken bone development

The skeletal system of birds stands out among other vertebrates due to their adaptation for active flight. These adaptive traits include pneumatic bones (except for forearm, metacarpus, and pelvic bones), no teeth, low bone marrow volume, corneous beak and large eye sockets compared to the whole skull. These adaptations have determined a low body weight in birds. The long bones include a spongy tissue which takes part in metabolic processes, is characterised by a low degree of calcification and subject to continuous remodelling, as well as by compacted tissue (Seifert and Watkins 1997). The avian skeleton is composed of few fractions, minerals (70%), organic compounds (20%) and water (10%; Turek 1984). Ca and P in the form of the hydroxyapatite account for over 90% of the mineral fraction (inorganic; Scott *et al.* 1982). The organic fraction of the osseous tissue is collagen fibres, primarily type I ones, although other types – like type V and XII – are known to be present (Burgeson 1988). Collagen fibres are mainly responsible for the elastic properties of bones. Collagen takes part in the formation and mineralisation of the osseous tissue. In addition, there are non-collagen fibres (Sommerfeldt and Rubin 2001) e.g. proteoglycans, osteocalcin, osteonectin, and adhesive proteins. Osteoblasts cells are responsible for the secretion of such proteins as osteonectin, osteocalcin and collagenase, which take part in the process of bone mineralisation, or secrete a protein called osteoprotegerin which binds with the RANKL glycoprotein, thereby preventing contact between osteoblasts and osteoclasts (Boyle *et al.* 2003).

The process of bone formation is referred to as osteogenesis. Bone is formed either from specific connective tissue or from cartilage. In the case of broiler chickens, researchers have described the process of osteogenesis from cartilage, because this process usually pertains to the lower limbs. Cartilage and its *lacunae* are formed due to cell hypertrophy at the site of Ca salt deposition, a process called calcification (Wuthier 1988). The process of bone formation is accompanied by the appearance of multiple osteoclasts on the surface of bone *trabeculae* which cause damage to both trabeculae and bone, thereby contributing to the formation of medullary (Bain and Watkins 1993). Bone formation in the secondary ossification centres appears later and is not accompanied by the formation of the bone collar. Once bone formation is completed in secondary ossification centres, articular cartilage covers the epiphysis and epiphyseal cartilage is formed at the epiphysis. The process of bone mineralisation may be described in four stages. Firstly, microcrystalline formation begins in the calcified cartilage, when dissolved ions of Ca and P form insoluble compounds. This leads to the formation of small molecules which – while the animal is growing – transform into the first hydroxyapatite crystals. At a subsequent stage, large crystals grow and proliferate, thereby forming an osteoid. The final stage involves the

formation and development of a structurally stable mineral fraction of the bone (Glimcher 1976). Figure 1 depicts markers of bone turnover processes, which connects two opposed processes in bone tissue i.e. bone modelling and resorption.

Calcium and phosphorus

Both Ca and P are the major macroelements found in living organisms, where they serve principally as building materials. In total, 99% of the Ca in the body is derived from the skeleton where, together with P, it forms hydroxyapatite (Bello *et al.* 2014). The skeleton provides the framework which supports the body and maintains its shape and protects fragile internal organs or tissues. Together with the attached muscles, it constitutes the locomotor system which allows animals to move. In addition, it is an indicator of the growth capability (Suttle 2010). A considerably lesser amount of Ca is found outside the skeletal system (*ca.* 1%). This part is indispensable for the maintenance of the proper functions of an animal. Either in the form of ions or other compounds, Ca is involved in multiple physiological processes. These include blood coagulation process, adhesion to various biomolecules, cell proliferation and differentiation, nervous signals transmission and muscle contraction (Mcdonald *et al.* 1995). Ca is responsible for the activity of enzymes which hydrolyse polysaccharides, phospholipids and proteins (Brody 1994). It affects viscosity of the cytoplasm, permeability of membranes, secretion of hormones and cell apoptosis. P is the second major element found mainly in bones (*ca.* 80%). Apart from bone formation and mineralisation, its biological functions include participation in the glucogenesis process as well as transport of fatty acids, amino acids and proteins. It is a constituent of nucleic acids that are necessary for cell differentiation and growth and phospholipids. Phospholipids aid the fluidity and integrity of cell membranes and contribute to the maintenance of both the osmotic and acid–base equilibrium (Suttle 2010). In addition, P plays a role in the transport and consumption of energy by AMP, ATP and ADP (Adeola *et al.* 2005).

The bone is subject to unceasing changes because its Ca and P are released *via* resorption to the blood stream and other sites in the body, e.g. soft tissues. This exchange of macroelements is of special significance to highly productive animals, like dairy cows or laying hens (Mcdonald *et al.* 1995), whereby the demand for Ca is several times higher than

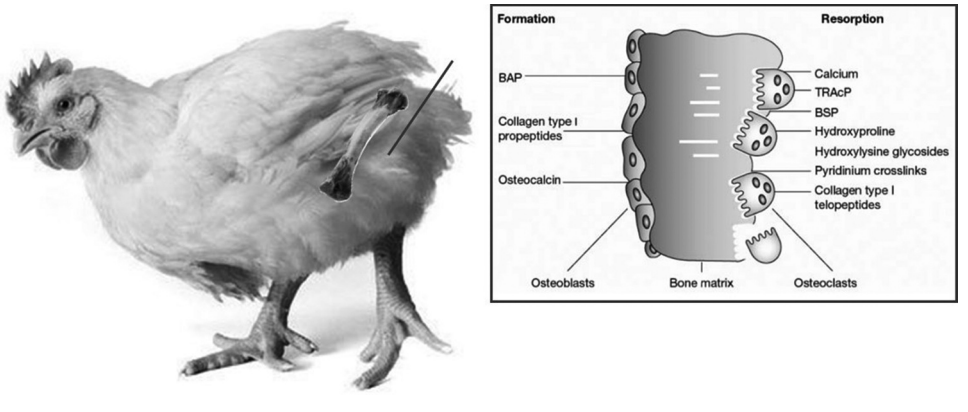

Figure 1. Markers of bone turnover in a bird's body (adapted from Seibel 2005).

in other animals. The Ca-P metabolism is regulated by hormones. The major two are the parathyroid hormone (PTH, referred to as parathormone) produced in parathyroid glands, and calcitonin which expresses the antagonistic action and is secreted by C cells of the thyroid gland. Parathormone is secreted by parathyroid glands during periods of hypocalcaemia, *i.e.* low calcium levels in blood serum. This hormone affects osteoclasts containing PTH-specific receptors, resulting in Ca release from the osseous tissue, thereby increasing concentration in blood serum. Parathormone influences renal functions as it increases Ca reabsorption in renal tubules, which leads to elevated serum level of Ca. In kidneys, it inhibits phosphate reabsorption. Furthermore, it participates in the synthesis of an active form of vitamin D3 (cholecalciferol), which plays an important role in the process of Ca absorption in the intestines (Frandson and Spurgeon 1992; Koreleski and Świątkiewicz 2005). In turn, calcitonin inhibits osteoclasts activity, which leads to bone resorption inhibition and reduced Ca release to blood. In the case of kidneys, calcitonin affects cells in the renal tubules, thereby blocking resorption of Ca and phosphates, which leads to their excretion (Hirsch and Baruch 2003; Martin *et al.* 2010). Recent investigations point to the influence of other factors on Ca and P metabolism, such as fibroblast growth factor 23 and Klotho proteins Klotho (Figure 2; Martin *et al.* 2012; Li, Zhang, and Bryden 2017b).

Requirements for broiler chickens

Nearly 25 years ago, the Nutrient Requirements of Poultry (NRC 1994) established poultry demands for Ca and available non-phytate P. It needs to be emphasised that earlier producers based their practice on demands for total P (NRC 1950), inorganic P (NRC 1954), and available P (NRC 1984), which did not reflect the real demand in birds. In the meantime, the genotype of broiler chickens has changed (Duclos *et al.* 2007), while no significant modifications were observed in their diets (Williams *et al.* 1998). Generally, diets for broiler chickens are supplemented with both minerals in their various forms (Waldenstedt 2006) to improve bone mineralisation and, consequently, to prevent diseases of the skeletal system.

The demand for minerals in animals, including poultry for these elements are usually treated together rather than separately, because Ca and P are interdependent. An excess or deficiency of one of them causes concomitant deficiency or excess of the other (Al. Masri 1995). A study conducted by Long *et al.* (1984) demonstrated that Ca administered in excess to broiler chickens caused the formation of insoluble Ca phosphates (Ca3(PO4) 2) in their intestines, which led to P deficiency. As reported by Bar *et al.* (2003), broiler chickens demand for P was similar in the context of bone mineralisation and proper growth, whereas there was a higher demand for Ca to meet the needs of mineralisation (calcification) than for body weight gains. The Ca to P ratio in broiler chicken diet is generally assumed at 1:1–2:1. In 1 kg of a starter type feed mixture, this corresponds to 10 g of Ca per 4.5 g of available P. Some authors reported a higher ratio of these elements in diets for broiler chickens, e.g. 2.6:1 (Williams *et al.* 2000). In previous work, these authors (Williams *et al.* 1998) proved that the Ca to P ratio should differ in feed mixtures for two-three week-old broiler chickens, depending on genotype. The ratio of these elements may be lower in the case of the slow-growing chickens (2:1) compared to the fast-growing chickens which need 3:1. Considering the maximum content of crude ash in bone, demands for Ca and P will differ depending on bird age. This has been discussed in

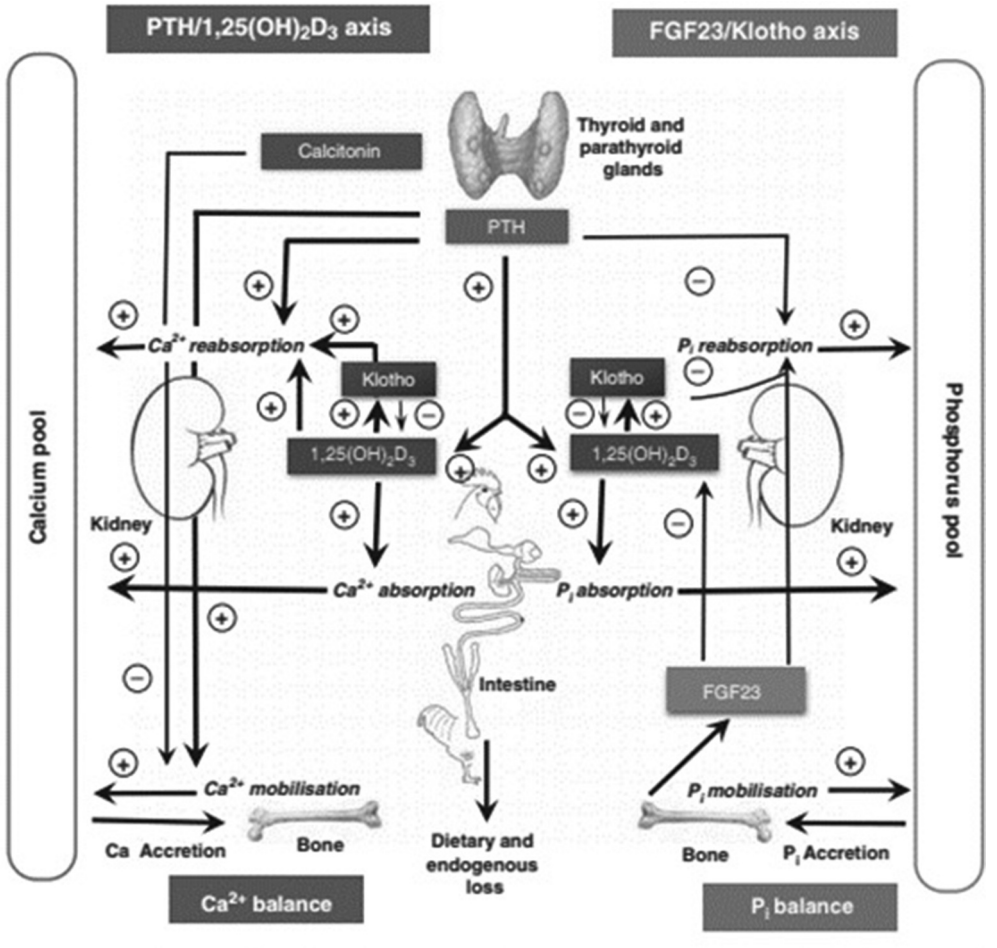

Figure 2. Metabolism of Ca and P is regulated through the concerned action of intestinal, skeletal and renal mechanism (adapted from Renkema et al. 2008; Li, Zhang, and Bryden 2017b).

ample works. Demands established for up to six week-old broiler chickens were 1.04% Ca and 0.36% available P (Huyghebaert 1996), for three to 30-d old birds were at 1.00% Ca and 0.44% available P (Rama Rao *et al.* 1999), whereas for one to 43-d-old birds were at 1.39–1.57% Ca and 0.48–0.57% available P (Bar *et al.* 2003). Recent investigations (Li *et al.* 2017a) showed that available P content of 3.5 g/kg and Ca content of 6.5 g/kg were more suitable in starter type mixtures administered in from one to 14 d of age in broiler chickens, with no phytase supplementation. Demands for Ca and P decreased during 15–21 d of age, reaching 3 g/kg and 6 g/kg, respectively. The same authors demonstrated that feed mixture supplementation with phytase contributed to improved performance in birds administered smaller doses of P (2–2.5 g/kg), and had no effect on bird's productivity upon the administration of higher P doses (3–3.5 g/kg). It seems that the broiler chickens tolerate a relatively wide range of Ca concentrations as far as their diet is concerned, as long as they receive sufficient concentrations of digestible P or phytase.

Equally important is the role of vitamin D_3 (cholecalciferol). Diet supplementation with vitamin D_3 is essential, considering its involvement in various biological processes, *e.g.* bone mineralisation or Ca and P absorption (Driver *et al.* 2005; Kasim *et al.* 2006). Increased absorption of these elements aided by cholecalciferol proceeds *via* stimulated production of Ca-binding proteins in the mucosa, which activates the Ca activated tenderisation (CAT) complex through the increase in plasma Ca (Santos 2006). It is difficult to explicitly establish the Ca:P ratio because of the various effects on their metabolism.

Sources and availability

Among the plant sources, only a few are rich in Ca, an exception being extracted rapeseed meal. It has been reported that *ca.* 20–30% of Ca found in plants occurs in the form of oxalates which are not absorbed by birds (Francesci and Nakata 2005). An additional limitation is the presence of phytic acid, found mainly in seeds, which impairs Ca absorption. Therefore, it is essential to supplement poultry diets with Ca using inorganic sources, including limestone consisting of ~97% of Ca carbonate ($CaCO_3$), and mono- and di-Ca phosphates, which are additionally sources of P in diet (Walk *et al.* 2012).

Limestone has a smooth structure and occurs in two forms: fine-grained and coarse-grained (most often used in poultry feeding). Certain alternative to the traditional Ca sources in feed mixtures may be offered from organic sources, usually characterised by a rough structure. An example is disintegrated oyster shells, as their Ca is absorbed by birds at almost 100%. Other sources are the shells of snails and clams (Ajakaiye *et al.* 2003; Rama Rao *et al.* 2006; Oso *et al.* 2011) or seaweed (Bradbury *et al.* 2016a). As demonstrated by Oso *et al.* (2011), the use of charcoal as a source of Ca diminished its availability and increased P content in the tibia. The mineral composition of selected Ca sources was presented in Table 1.

In the case of P, the major sources in feedstuff for poultry include cereal seeds and by-products from oil production or cereals processing. Relatively high contents of this element may be found in extracted soybean or rapeseed meals, *i.e.* approximately 4–15 g/kg dry matter (Jeroch 1994). In feedstuff materials, *ca.* 50–90% of P is in the phytate form (Oloffs *et al.* 2000) bound in a complex (Selle and Ravindran 2007; Wilkinson *et al.* 2011). Acquisition of this element from phytic compounds requires the presence of the enzyme phytase, which induces hydrolysis in the gastrointestinal tract of birds. Chickens are capable of synthesising small amounts of this enzyme (Maenz and Classen 1998), which, in practice, means that it lacks endogenous phytase. The microbiome of the gastrointestinal tract of birds is incapable of producing sufficient amounts of

Table 1. Contents of Ca and P in selected organic and inorganic sources (based on Oso *et al.* 2011).

Source	Ca [g/kg]	P [mg/kg]
Oyster shells	379.6	50.1
Charcoal	218.1	180.0
Snail shells	332.1	170.0
Limestone	357.61	160.1

this enzyme (Marounek *et al.* 2008). Among natural plant sources, only certain cereals, in particular rye, triticale or wheat, contain phytase which is able to release P from a phytin molecule (Cossa *et al.* 1999). However, phytase activity in cereals varies (Oloffs *et al.* 2000) and depends on species and processing treatments including granulation or re-drying and storage. Therefore, commercial phytases sourced from *Aspergillus* fungi and/or bacteria (Jayaprakash *et al.* 2016), have been used for years in poultry feed. Inorganic phosphates have been widely used as supplements (Applegate and Angel 2008), including mainly mono-, di-calcium, defluorinated or magnesium phosphates. Feed mixtures usually contain mixtures of mono- and di-calcium phosphates (Viljoen 2001). Their composition differs slightly, for example monocalcium phosphate contains 22.7% Ca by weight and 18% P by weight. In turn, dicalcium phosphate contains 18% and 24% by weight of the hydrated form as well as 20–21% and 25–29% by weight of the anhydrous form (Grochowicz 1996). Considering this, the Ca to P ratio is higher in dicalcium phosphate. Today, however, the use of phosphates is limited due to environmental concerns and supplementation with phytase, which improves P absorption from plant components (Swick and Ivey 1990; Ptak *et al.* 2013).

Another important issue is Ca solubility in the acidic environment of the gastrointestinal tract prior to absorption. Calcium carbonate, in the form of limestone, being the most frequently used Ca source in poultry feeding, is approximately 80% soluble and absorbable in the acidic medium of the gastrointestinal tract (Walk *et al.* 2012). This suggested that Ca solubility is most effective in the upper sections of the gastrointestinal tract, namely in the ventriculus (gizzard) and proventriculus (pH 1.5–4, Svihus 2011), compared to the small intestine where the pH is higher. Alternative sources of Ca could be used to offer better digestibility.

The second aspect is the size of Ca particles derived from various sources. Early investigations addressing this issue have been mainly undertaken in the context of laying hens (Scott *et al.* 1971; Rao and Roland 1990; Skrivan *et al.* 2010). Calcium solubility in the gastrointestinal tract of chickens is related to the formation of complexes with phytate P. As reported by Manangi and Coon (2007), chickens fed phytase could better absorb Ca from limestone with greater particle sizes and poorer solubility. Larger particles do not dissolve in the crop nor in the anterior segments of the gastrointestinal tract (ventriculus and proventriculus). The forms of Ca carbonate with a poorer solubility may lead to more effective hydrolysis of phytate forms of P by phytase in the intestines. The passage of smaller particles of Ca carbonate is characterised by better solubility throughout the gastrointestinal tract (Manangi and Coon 2007). Calcium ions derived from small particles may form mineral-phytate complexes which impair the capability of exogenous phytase for hydrolysis. An early study with broiler chickens demonstrated that the use of medium-sized Ca particles (0.25–0.85 mm) yielded better results for crude ash content of bones compared to larger, more rough particles (2.36–3.35 mm; Mcnaughton *et al.* 1974). Other investigations with broiler chickens showed that birds administered medium-size Ca carbonate particles in their diet (from 137 to 388 µm) attained higher final body weights and ash content of the tibia compared to birds administered smaller particles at *ca.* 28 µm and the largest particles at 1306 µm (Manangi and Coon 2007). Other authors suggested that highly soluble Ca sources contributed to the improvement of production parameters and bone mineralisation (Walk *et al.* 2012). Very soluble sources of Ca and phytase allowed a reduction in the amount of supplemented Ca. This led to concern over

excess amounts of Ca included in feed. The use of exogenous phytase with concomitant use of two Ca sources, i.e. limestone and calcified seaweed (an alternative source), at 5.5 g/kg improved Ca digestibility, which was dependent on Ca source and particle size. Better Ca digestibility was observed for limestone particles larger than 0.5 mm and for calcified seaweed particles smaller than 0.5 mm (Bradbury *et al.* 2016b). The results indicated that Ca particle size recommended for laying hens and broiler chickens depends on bird age. But in the case of broiler chickens, the concentration, source or particle size of Ca have a lesser effect on their production results.

Nanoparticles as sources of ca and p in broiler diets

There are multiple examples for the use of nanoparticles, like for instance diagnostics and detection of pathogens or proteins (Kaittanis *et al.* 2010), transport of drugs and tissue engineering (Shi *et al.* 2010), cancer studies (Salata 2004; Wierzbicki *et al.* 2017) and antimicrobial properties (Duncan 2011). Nanoparticles are useful in poultry production (Fisinin *et al.* 2018; Abd El-Ghany 2019) and the current research has focussed *e.g.* on silver nanoparticles (Sawosz *et al.* 2012; Chauke and Siebrits 2012; Sawosz *et al.* 2013; Ahmadi *et al.* 2013; Elkloub *et al.* 2015), selenium (Fuxiang *et al.* 2008; Wang 2009; Zhou and Wang 2011; Cai *et al.* 2012; Mohapatra *et al.* 2014;; Selim *et al.* 2015; Shirsat *et al.* 2016), copper (Wang *et al.* 2011; Pineda *et al.* 2013; Mroczek-Sosnowska *et al.* 2015; Scott *et al.* 2016; Sawosz *et al.* 2018; Sizova *et al.* 2019) and more combinations with another nutrients (Yausheva *et al.* 2016).

According to Swain *et al.* (2015) nanoparticles have great potential even at very low doses compared to conventional inorganic or organic sources of minerals including Ca and P. Due to the continuous search for alternative sources of macrominerals which may be more efficiently used by birds due to, for example, their size, nanotechnology has aroused increasing interest. Recent studies have addressed nanoparticles of Ca-P in feed, including Ca phosphate (Vijayakumar and Balakrishnan 2014, 2015; Samanta *et al.* 2019) and dicalcium phosphate (Hassan *et al.* 2016; Mohamed *et al.* 2016), Ca carbonate (Salary *et al.* 2017) and hydroxyapatite (Sohair *et al.* 2017). In investigations, nanoparticles have been perceived as alternatives to traditional sources of Ca and P in feed. A general aim was to reduce the use of large amounts of selected sources of elements through the application of more highly bioavailable forms due to effective absorption (Gonzales-Eguia *et al.* 2009). Initial poultry reports concerned the effect of Ca phosphate nanoparticles. There were no significant negative changes in levels of biochemical blood markers, including glucose, albumins, triglycerides, cholesterol, creatinine or selected hepatic enzymes, with use of Ca phosphate nanoparticles compared to broiler chickens fed traditional dicalcium phosphate (Vijayakumar and Balakrishnan 2014). Similar observations were made for selected haematological parameters (Vijayakumar and Balakrishnan 2015). A study conducted by Mohamed *et al.* (2016) showed no significant changes in the mineral content of the gizzard, heart, and liver. Diets formulated with only 25% of the required non-phytate P derived from nanoparticles of dicalcium phosphate could be used instead of diets containing 100% demand for this element from conventional dicalcium phosphate. The same authors observed higher final body weight, better feed conversion ratio and higher daily weight gains in groups of birds fed diets with nano dicalcium phosphate. Other authors observed decreased Ca and P excretion in faeces (by *ca.* 50%) in birds fed

nanoparticle sources (Hassan *et al.* 2016). Vijayakumar and Balakrishnan (2015) demonstrated no significant differences in the morphometry and tibia mineralisation upon the use of 100% dicalcium phosphate (control group) as a source of Ca and P and with increasing inclusion of dicalcium phosphate nanoparticles instead the conventional form (from 50 to 100% nano form). This was confirmed by 200% better bioavailability of P and Ca from nanoparticles than from the conventional dicalcium phosphate. Findings from these investigations may have some measurable effects on environment protection. The study of Samanta *et al.* (2019) demonstrated that the application of Ca phosphate nanoparticles in different levels, both as the only source and in combination with standard Ca phosphate, did not affect cumulative feed consumption or feed conversion ratio, biochemical parameters or carcase characteristics. However, the authors reported that the growth performance was better in birds fed nanoparticles at the 50% level. In a study with hydroxyapatite nanoparticles, authors showed that this form could be an adequate source of Ca and P in broiler feed. Nano hydroxyapatite led to improve body weight gain and feed intake (by 6% and 8% with nano hydroxyapatite, respectively) but did not affect FCR in birds in comparison with the control group fed dicalcium phosphate at levels of 2% of the diet. The fact which should be underlined is that the results indicated that the Ca and P from nano hydroxyapatite were considerably better absorbed and subsequently reduced in excreta (Sohair *et al.* 2017). Application of Ca carbonate through *in ovo* injection resulted in higher body weight and some organ weights (spleen, bursa of fabricius) in broiler chicken hatchlings (Salary *et al.* 2017). In the same study, broiler chickens reared to 21 days post-hatch, did not differ in weight gain, FCR and feed intake between groups.

Studies conducted so far have mainly been focused on the administration of various nanoparticles in water or in feed for poultry to improve the health status of birds or absorption of minerals, including administration *via in ovo* injections. Little work has addressed the use of nanoparticles of Ca and P on the skeletal system. Mohamed *et al.* (2016) compared effects of dicalcium phosphate as nanoparticles and in a conventional form on selected traits of carcass and other important parameters, i.e. quality of long bones, in 26-day-old broiler chickens. Interestingly, dicalcium phosphate nanoparticles had a positive impact on morphometric measurements of the tibia, including weight, length, width and breaking force, when used in a significantly lower dose compared to the conventional form. Bone development was affected by *in ovo* application of Ca carbonate nanoparticles, resulting in significantly higher Ca and Cu, but not P, concentrations in tibia bones in broiler hatchlings. These authors demonstrated that the activity of alkaline phosphatase in serum increased significantly in groups injected *in ovo*. Alkaine phosphatase plays an important role in ossifying and calcification of the bones. Further studies at this context should be performed, especially for Ca and P nanoparticles.

Conclusions

Calcium and P are key elements in the skeletal mineralisation process. Therefore, appropriate supplementation is of utmost significance, especially in the case of highly productive animals, such as broiler chickens. The excessive use of Ca carbonate and phosphates together with phytase in feed is common practice today, usually due to incomplete absorption of Ca and P from these sources. This gives rise to serious concerns regarding the effects of excreted elements on the natural environment. An alternative in

this case seems to be the implementation of alternative Ca and P sources in the form of nanoparticles. Investigations conducted so far are highly prospective and afford the possibility for reducing the inclusion levels of these elements.

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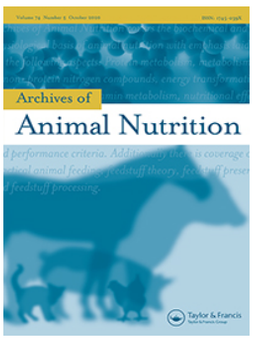

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Effect of *in ovo* application of hydroxyapatite nanoparticles on chicken embryo development, oxidative status and bone characteristics

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ABSTRACT

Intensive selection in modern lines of fast-growing chickens has caused several skeletal disorders. Therefore, current research is focused on methods to improve the bones of birds. A new potential solution is *in ovo* technology using nanoparticles with a high specificity for the bone tissue. Thus, the objective of the present study was to evaluate the effect of *in ovo* application of hydroxyapatite nanoparticles (HA-NP) in different concentrations (50, 100 and 500 µg/ml colloids) on chicken embryo development, with a particular focus on the oxidative status and bone characteristics of the embryo. The results showed that *in ovo* treatment with HA-NP did not negatively affect hatchability and body weight. However, bone weight was reduced in 500 µg/ml group. The concentrations of calcium, phosphorus and crude ash were not affected. The modulatory effect of HA-NP was observed on the basis of antioxidative markers – superoxide dismutase, total antioxidant status, malondialdehyde in serum and selected tissues. Glutathione concentration in serum suggested higher metabolic stress. Among bone turnover markers, the concentration of osteocalcin was found to be significantly affected by HA-NP injection. Thus, the *in ovo* application of HA-NP could modify the molecular responses at the stage of embryogenesis but these changes were not reflected in embryo growth and even slowed down bone development. Nevertheless, the question for the follow-up research is whether *in ovo* administration of HA-NP would affect the antioxidative status and bone turnover resulting in improved bone conditions and body gain in post hatch chickens.

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1. Introduction

The increase in body weight gain of broilers by almost 300% within the last 50 years is an effect of an intensive selection for this parameter (Petracci and Cavani 2012). Modern

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commercial broiler lines are bred to maximise the feed conversion ratio and growth rate in a very short period of time. However, this rapid weight gain has also been the main cause of leg pathologies such as deformations or osteoporosis in broiler chickens (Fleming 2008).

Calcium plays a role in various physiological processes, for example, blood coagulation, cell proliferation, and muscle contraction (McDonald et al. 1995). It is also involved in the activation of enzymes, affects the permeability of membranes, the viscosity of the cytoplasm and hormone secretion. Phosphorus is involved in the gluconeogenesis, transport of amino acids, proteins and fatty acids. It plays a major role in biological molecules for example by forming part of nucleic acids. It takes part in cell growth and differentiation, being used by cells to transport energy in the form of ATP. P is a part of phospholipids which are the main structural components of cellular membranes (Suttle 2010). Ca and P are the main contributors to the mineral structure of the bone where they occur in the form of hydroxyapatite. Moreover, 99% of Ca in the body is derived from the skeleton (Turek 1984; Bello et al. 2014). It is well known that the intake of Ca and P and the appropriate ratio of these elements are necessary nutritional factors to ensure bone strength (Scott et al. 1982) and compounds of these minerals in nanoparticle form are highly prospective in poultry nutrition (Matuszewski et al. 2020).

Bone is formidable for its resilience, harness and regenerative capacity. It provides rigorous support and protection to the soft parts of the body and furnishes a system for muscle growth (Turek 1984). Similar to other birds, most of the skeleton in chickens is formed as cartilage, which later becomes ossified. Bones formed in this way are well known as cartilaginous bones. They are different from membranous bones that are ossified from mesodermal tissue (Schepelmann 1990). The woven bone is present in the first stages of embryonic development and is characterised by immaturity, osteocyte content and irregular collagen fibres. During the growth and development stage, the woven bone, as a temporary bone, transforms into the lamellar bone (Adler 2000). The process of bone mineralisation may be described in four stages: microcrystalline formation in calcifying cartilage, formation of hydroxyapatite crystals, formation of the osteoid from larger crystals, and development of structurally stable mineral fraction (Glimcher 1976). The bone modelling process is strictly associated with biochemical bone turnover markers such as bone-specific alkaline phosphatase (BALP), osteocalcin (OC), procollagen type I N-terminal propeptide (PINP) and osteoprotegerine (OPG). These markers are synthesised in osteoblasts and play various roles. Some of them are commonly used as markers in the prediction of fracture risks and in the diagnosis of osteoporosis in humans by indicating the level of bone mineralisation (Vasikaran et al. 2011; Wheater et al. 2013). A noteworthy fact is that the fastest development of the chicken occurs during the first few days of age.

It is well known that residual yolk is the main source of nutrients for the developing chicken embryo (Henderson et al. 2008; Gonzales et al. 2013). Hence, the yolk sac may lack nutrients essential for bone development, especially for embryos of high productive birds such as broiler chickens. From the genetic point of view, fast-growing genotype embryos during development use energy, lipid, protein and mineral resources that were deposited in the egg from adult birds (Ghobadi and Matin 2015), however, these may not be sufficient to cover requirements (Sawosz et al. 2012). Low consumption during incubation could be satiated by the *in ovo* injection

(IOI) of additional nutrients (Salary et al. 2014) for a better post-hatch growth. *In ovo* technology still remains an interesting area of research as an alternative method of functional nutrition, using various compounds. IOI of amino acids, minerals, carbohydrates, plant extracts and vitamins showed that this technique may positively influence hatching conditions, immune status, gut morphology and organs weight (Selim et al. 2012; Gonzales et al. 2013; Yair et al. 2013; Saki and Salary 2015; Zhang et al. 2018). Nutrient supplementation by IOI could be more efficient when a compound is attached to nanoparticles, which can deliver it inside the cells due to their small size, shape, a very high surface to volume ratio and physical activity. Nanoparticles administered directly to the egg, already at the stage of development of the chicken embryo, can lead to a number of systemic changes (Sawosz et al. 2012; Zielinska et al. 2012; Mroczek-Sosnowska et al. 2017; Scott et al. 2018). On the other hand, especially IOI of insoluble compounds of minerals can potentially initiate detoxification which, at the same time, may put higher metabolic stress in embryos.

In recent years, biomaterials in general and bone-related implant materials in particular have been significantly refined (Huebsch and Mooney 2009). Hydroxyapatite is a naturally occurring mineral form of calcium apatite. Bone mineral, a modified form of hydroxyapatite, constitutes up to 50% by weight of human bone (Junqueira and Carneiro 2002). Hydroxyapatite nanoparticles (HA-NP) are the subject of studies in the context of their potential for application in bones. HA-NP are in the same size range as integral parts of natural bone, because of which they are promising for local applications. HA-NP can form the basis of modular systems, which provides the opportunity to induce cell responses in a spatially and temporally controlled manner due to defined release of physiologically active substances (Biondi et al. 2008). Several studies on the interactions of HA-NP with osteoblasts showed the differences depending on the shape or size of HA-NP. Needle-shaped and spherical HA-NP decreased osteoblast cell numbers as shown by Xu et al. (2009). On the other hand, in another study, HA-NP with a larger diameter of 80 nm had no effect on the growth of osteoblast-like cells when compared with HA-NP with a smaller diameter of 20 nm. It has also been demonstrated that compared to conventional microcrystalline hydroxyapatite promotes osteoblast adhesion, proliferation, differentiation and osteointegration (Webster et al. 2000) due to osteoconductive properties (Gamagedara and Rajapakse 2019). HA-NP could be potentially used as a bone-building supplement with greater absorption than Ca, similar to microcrystalline hydroxyapatite possessing this feature (Straub 2007).

This part of a project consists of measurements with embryos, while the next part will elucidate effects of *in ovo* injection of hydroxyapatite on performance and bone health after hatching to 42 d of life. Therefore, in the present study it was hypothesised that embryonic supplementation of HA-NP (particle and ion forms) at early stage of incubation could cause a stimulative and modulatory effects on the chicken embryo development with special regard of the bone. HA-NP due to the nanometric size conditioning easy uptake by cells, can modulate bone mineralisation and thus help in bone formation in the pre- and post-embryonic life of birds. The objective of this investigation was to evaluate the effect of the *in ovo* application of HA-NP on chicken embryo development, oxidation status and bone characteristics.

2. Materials and methods

2.1. Experimental design

The experimental material consisted of 120 chicken eggs from 37-week-old Ross × Ross 308 hens obtained from a commercial certified hatchery. All eggs were laid at the same day, and represented the same weight class. The eggs were stored in a refrigerator at 12°C and 73% humidity for 2 d and then placed in an incubator (Jamesway, Canada). On d 1 of incubation, the eggs were weighed (56.4 ± 1.78 g) and randomly divided into four groups (4 × 30 eggs): no injection (control) and three groups with injection of HA-NP hydrocolloids in different concentrations. Before injection, the eggs were immersed in 0.5% solution of potassium permanganate, and 0.2 ml of the experimental hydrocolloids was then injected under sterile conditions into the albumen by using 27-gauge, 20-mm needles. Immediately after the injection, the hole was sealed using a sterile tape, and the eggs were placed in an incubator. The eggs were incubated for 20 d under standard conditions (temperature 37.8°C, humidity 55%, turned once per hour for the first 18 d; at 37°C and 75% humidity on d 19 and 20). During incubation, the eggs were two times exposed to light on the 7^{th} and 18^{th} d to control the accuracy of embryo development and to avoid embryo death. According to II Local Ethics Committee for Animal Experiments in Warsaw University of Life Sciences, tests on chicken embryos do not require the consent of the Committee.

On d 20, all embryos were weighed and decapitated. During decapitation, 10 samples of blood (one sample respresents pooled blood from two embryos) from the jugular artery from randomly selected embryos per group were collected into glass tubes, stored at 4°C for 2 h and then centrifuged for 5 min at 1200 · *g* (MPW-350 R centrifuge, MPW Med. Instruments, Poland). The obtained serum was stored in cryovials at −80°C. After blood collection, randomly selected embryos (n = 10 per group) were immediately transferred on dry ice, then, after cooling, livers, breast muscles, femurs and tibias were collected, weighed, and then stored at −80°C. Samples of serum from each group were analysed by standard laboratory procedures in the Veterinary Diagnostic Laboratory at Warsaw University of Life Sciences, using commercial diagnostic kits.

HA-NP (nanopowder, 98.5%) were obtained from SkySpring Nanomaterials, Inc. (Texas, USA). Hydrocolloids of HA-NP of different concentrations (50, 100, and 500 μg/ml) were produced by the electric nonexplosive high voltage method from high purity metals (99.99%) and high purity demineralised water. The process of hydrocolloid formation and the prevention of aggregate formation were supported by mixing and ultrasound activity. After premixing the whole solution for 5 min, ultrasound was introduced for 45 min by using Ultron U 509 apparatus (Transfer Multisort Elektronik, Lodz, Poland). Average particle size and zeta potential measurements were carried out using Zetasizer Nano-ZS ZEN 3600 (Malvern Instruments Ltd., Malvern, UK) with the dynamic light scattering mode and laser Doppler electrophoresis, respectively, at room temperature (23°C). The size and shape of HA-NP were visualised using a Morgagni 268D transmission electron microscope with a wide-angle Olympus Morada digital camera (FEI, Oregon, USA).

2.2. Determination of selected indicators of redox state

Ten samples of 250 μl of serum were used for the determination of malondialdehyde (MDA) level according to the method proposed by Kapusta et al. (2018). First, 25 μl of

0.2% 2,6-bis(1,1-dimethylo)-4-methylphenol (BHT, in ethanol) and 1 ml of 5% trichloroacetic acid (aqueous, TCA, Merck, Warsaw, Poland) were added to each sample and vortexed. After centrifugation at 14,000 · *g* for 10 min, 750 µl of supernatant was transferred to a glass tube, and 500 µl of 0.6% thiobarbituric acid (aqueous, Merck) was added, mixed, and incubated for 45 min in a water bath at 90°C. The supernatants were then stored in cool conditions and then centrifuged at 4000 · *g* for 5 min. Then, 100 µl of clear supernatant was transferred into a microplate. MDA concentration was determined using Tecan's NanoQuant Infinite M200 PRO (Tecan Austria GmBH, Grödig, Austria) analyser at the wavelength of 532 nm.

Glutathione (GSH) was determined quantitively on the basis of Ellman's method modified by Matusiewicz et al. (2019), in which 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB, Ellman's reagent) is reduced by thiol compounds to form a coloured product (2-nitro-5-mercaptobenzoic acid) with the maximum absorbance at 412 nm. To 375 µl of samples of serum from each group, 19.7 µl of 50% TCA was added and centrifuged (1200 · *g*, 5 min). Then, 6.25 µl of deproteinized supernatants were transferred into a microplate and mixed with 50 µl of 0.2 M phosphate-buffered saline (PBS) and 6.25 µl of $6 \cdot 10^{-3}$ M DTNB. The absorbance was measured using Tecan's NanoQuant Infinite M200 PRO (Tecan Austria GmbH) analyser.

Oxidation markers were measured in soft tissue but not in serum as there was not enough serum from embryos to perform all molecular analyses. The activity of superoxide dismutase (SOD), glutathione peroxidase (GSH-PX), total antioxidant status (TAS) and glutathione reductase (GR) was determined in the liver and breast muscle. Six samples of each tissue of approximately 200 µg from each group were added to 1 ml of PBS in a 2-ml Eppendorf tube and homogenised using the rotary hand homogeniser. The protein level in samples was determined using the Bicinchoninic Acid Kit (BCA, Merck). Then, 25 µl of homogenised supernatants were transferred into a microplate in two replicates, and 200 µl of reagent mixture was added. The analyser wavelength was 562 nm. The activation of the enzymes SOD and GSH-PX in the selected tissues of embryos was determined by spectrometry using Ransod and Ransel diagnostic kits manufactured by Randox (Poland). GR and TAS levels were determined using diagnostic kits manufactured by Randox.

2.3. Bone measurements

Left and right femurs and tibias obtained after decapitation were used for further analysis. Ten left and right femurs and 10 left and right tibias were carefully cleaned, and their length, diameter in the middle of the shaft, and diameter of the proximal epiphysis were measured using an electronic calliper for linear measurements. Subsequently, the breaking force of all bones was determined using a Zwick testing machine (Z0.5 Zwicki-Line, Ulm, Germany) with a warhead equipped with a Warner-Bratzler blade with a maximum force of 1 kN. The blade movement speed was 50 mm/min. Then, the cuts from the epiphysis area from left femurs (one replicate per group) were prepared for scanning electron microscopy imaging. Six left femurs were initially grounded in a mortar, suspended in 1 ml of PBS and homogenised. Six right femurs and tibias were weighed on an analytical balance in quartz beakers (weighing about 0.1 g), smoked on a hot plate (max. temp. 400°C) and burned in a muffle furnace with temperature control at about 470°C for 36 h. After cooling, 2 ml of redistilled water and HCl (38%) were added to the samples and transferred quantitatively with 40 ml of

redistilled water. The mineral contents (Ca and P) of the bones were determined by ICP-AES Thermo iCAP 6500 DUO (Thermo Scientific, USA) atomic emission spectrometer. The method consists of measuring the intensity of the radiation emitted in the solutions of the tested samples (characteristic for a given element line), which is a measure of the concentration of the determined element. The result of the analysis was within the range of the calibration curve. Calibration standards were prepared from certified standards at a concentration of 1000 ppm. Calibration range for Ca was 0–150 ppm and for P 0–100 ppm. The value was given as a mean concentration of the selected element at following wavelengths: for Ca – 316; 318; 373; 422 nm and for P – 177; 178; 179; 213 nm.

2.4. Determination of selected bone turnover markers

To evaluate the selected bone turnover markers in embryo's serum, 10 samples of serum from each group were used. The concentrations of BALB, PINP, OC and OPG were measured by chicken-specific enzyme-linked immunosorbent assay (ELISA) kits obtained from Immunogen, Poland (catalogue numbers: MBS738109, MBS268643, MBS2512530, and MBS261019). The homogenates from femur bones were used to determine OC and PINP concentrations in conversion to bone protein by using the same ELISA kits. The protein concentration was measured by the method described above. According to the assay protocols, the absorbance was measured using Tecan's NanoQuant Infinite M200 PRO (Tecan Austria GmbH) analyser at 450 nm.

2.5. Statistical analysis

For the analyses, individual embryos were treated as experimental units. We used the minimum necessary sample size according to our previous studies (Pineda et al. 2013; Łukasiewicz et al. 2020). The collected data were subjected to statistical analysis using the general linear model of one-way analysis of variance – ANOVA. The significance of differences between the groups was evaluated using Tukey's test (IBM SPSS Statistics version 21.0). The level of significance was set at $p \leq 0.05$.

3. Results

3.1. Physicochemical properties of HA-NP

The average zeta potential was −12.3 mV, indicating the stability of the solutions HA-NP were characterised by spherical shape. The diameter of single NP was in the range of 20–35 nm and the average size of agglomerate was 404 nm (Figure 1).

3.2. Hatchability and embryo development

The results indicated that HA-NP at different concentrations injected *in ovo* did not affect the hatchability (Table 1). All embryos showed normal growth and development, and no defects were observed. Furthermore, Table 1 presents body and selected organs weights. It was observed that IOI of HA-NP did not affect weight of body, muscle and liver (except at 100 HA-NP).

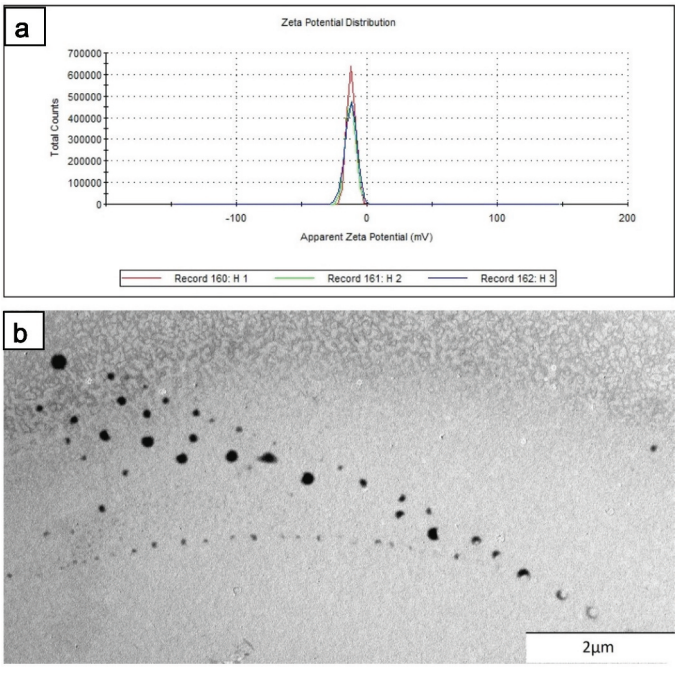

Figure 1. Representative zeta potential of HA-NP at the concentration of 50 µg/ml (three peaks) (A). Transmission electron microscopic image of HA-NP; scale bar represents 2 µm (B).

Table 1. Hatchability, body and selected organ weights of chicken embryos on d 20 after *in ovo* treatment with hydroxyapatite nanoparticles (HA-NP).

	Hatchability [%]	Body weight [g]	Liver weight [g]	Breast muscle weight [g]
500 HA-NP[†]	93	43.6	0.94[AB]	0.78
100 HA-NP[‡]	93	43.4	0.84[B]	0.74
50 HA-NP[§]	90	43.3	1.06[A]	0.73
C[¶]	93	43.3	1.01[A]	0.72
SEM[#]	-	0.61	0.012	0.009
p-Value	-	*ns*[*]	*<0.001*	*ns*

[+]500 HA-NP, eggs injected with 500 µg HA-NP/ml colloid; [±]100 HA-NP, eggs injected with 100 µg HA-NP/ml colloid; [§]50 HA-NP, eggs injected with 50 µg HA-NP/ml colloid; [¶]C, control group, not injected; [#]SEM, standard error of the mean; [*]ns, non-significant. [A,B,C]Means not sharing the same superscript differ significantly at $p \leq 0.05$ ($n = 10$ per group).

3.3. Bone measurements

The selected parameters for both types of bones are shown in Tables 2 and 3. The weight of tibia and femur was affected by injection of HA-NP. Tibia weight in the 500 HA-NP group was significantly lower than in the other groups. A similar situation was observed in femur weight, which was the lowest in the 500 HA-NP group. The length of tibia was not affected, but femur was only reduced in 100 HA-NP group. The breaking strength of tibia and femur were not different between all groups. Scanning electron microscope images suggested the normal development of bones, with low porosity (Figure 2).

Table 2. Effect of *in ovo* treatment with hydroxyapatite nanoparticles (HA-NP) on morphometric measurements and breaking strength of tibias from chicken embryos on d 20 of treatment.

Group	Bone weight [g]	Bone diameter [mm]	Bone length [mm]	Epiphysis diameter [mm]	Breaking strength [N]
500 HA-NP[†]	0.22[B]	1.80	29.4	5.06	16.2
100 HA-NP[‡]	0.28[A]	1.78	29.8	4.82	16.5
50 HA-NP[§]	0.29[A]	1.79	29.8	5.07	16.8
C[¶]	0.28[A]	1.75	30.2	4.82	15.6
SEM[#]	0.009	0.029	0.28	0.117	1.07
p-Value	*<0.001*	*ns**	*ns*	*ns*	*ns*

[†]500 HA-NP, eggs injected with 500 µg HA-NP/ml colloid; [‡]100 HA-NP, eggs injected with 100 µg HA-NP/ml colloid; [§]50 HA-NP, eggs injected with 50 µg HA-NP/ml colloid; [¶]C, control group, not injected; [#]SEM, standard error of the mean; [*]ns, non-significant. [A,B]Means not sharing the same superscript differ significantly at $p \leq 0.05$ ($n = 10$ per group).

Table 3. Effect of *in ovo* treatment with hydroxyapatite nanoparticles (HA-NP) on morphometric measurements and breaking strength of femurs from chicken embryos on d 20 of treatment.

Group	Bone weight [g]	Bone diameter [mm]	Bone length [mm]	Epiphysis diameter [mm]	Breaking strength [N]
500 HA-NP[†]	0.17[B]	1.74	21.5[A]	4.40[B]	18.9
100 HA-NP[‡]	0.18[A]	1.82	21.4[B]	4.65[A]	16.8
50 HA-NP[§]	0.18[A]	1.83	21.8[A]	4.74[A]	20.4
C[¶]	0.19[A]	1.76	22.0[A]	4.61[A]	18.3
SEM[#]	0.005	0.025	0.26	0.083	1.54
p-Value	*<0.01*	*ns**	*<0.01*	*<0.01*	*ns*

[†]500 HA-NP, eggs injected with 500 µg HA-NP/ml colloid; [‡]100 HA-NP, eggs injected with 100 µg HA-NP/ml colloid; [§]50 HA-NP, eggs injected with 50 µg HA-NP/ml colloid; [¶]C, control group, not injected; [#]SEM, standard error of the mean; [*]ns, non-significant; [+]AST, aspartate aminotransferase; [$]ALT, alanine aminotransferase; [!]ALP, alkaline phosphatase; [◊]LDH, lactate dehydrogenase; [♦]MDA, malondialdehyde. [A,B]Means not sharing the same superscript differ significantly at $p \leq 0.05$ ($n = 10$ per group).

Figure 3 shows percentage mineralisation of both types of bones. The results did not show any significant differences between the groups.

3.4. Serum biochemical parameters

The results of biochemical parameters of the serum of chicken embryos indicated no significant differences between the groups and indicate the normal liver and kidney functions (Table 4).

3.5. Indicators of redox state in serum and tissues

Table 5 shows the selected parameters related to oxidation status. The MDA content was affected by IOI of HA-NP, especially for a significant difference between the control and 500 HA-NP group which was characterised by the lowest value of MDA. The IOI of HA-NP at different concentrations affected GSH concentration in serum ($p < 0.05$), which was the lowest in control. The levels of SOD, GSH-PX, TAS and GR were determined in the liver and muscles of the embryos. The SOD concentrations were significantly different in both breast muscle and liver. It was the highest in the breast muscle in the control group, being significantly different from 100 HA-NP and 50 HA-NP groups. In

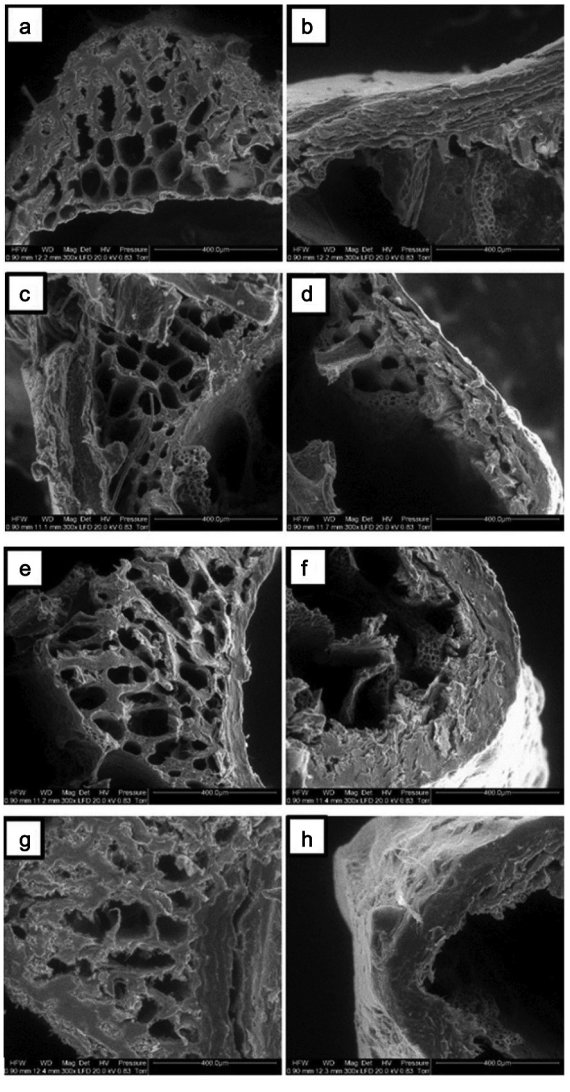

Figure 2. Scanning electron microscope images of femur bones in experimental groups: 50 µg/ml (A,B), 100 µg/ml (C, D), 500 µg/ml (G, H) and control (g,h). Scale bar represents 400 µm.

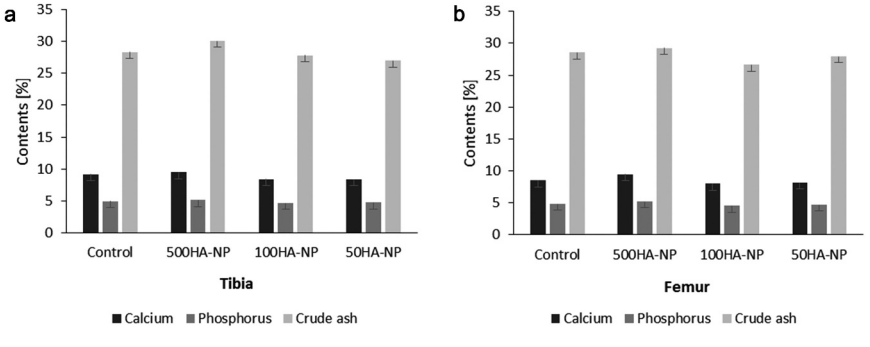

Figure 3. Calcium, phophorus and crude ash content of tibia (A) and femur (B) of chicken embryos on d 20 after *in ovo* treatment with HA-NP.

Table 4. Results of selected parameters in the blood serum of chicken embryos on 20 d after *in ovo* treatment with hydroxyapatite nanoparticles (HA-NP).

	Group					
	500 HA-NP[†]	100 HA-NP[‡]	50 HA-NP[§]	C[¶]	SEM[#]	*p*-Value
AST[+] [U/l]	195	165	301	280	12.0	*ns**
ALT[$] [U/l]	7.80	11.60	4.83	4.23	0.67	*ns*
ALP[‖] [U/l]	920	692	838	752	27.8	*ns*
LDH[◊] [U/l]	1041	981	839	1129	37.3	*ns*
Glucose [mmol/l]	282	259	256	257	1.99	*ns*
Creatinine [mmol/l]	0.36	0.33	0.35	0.36	0.001	*ns*
Total protein; [mmol/l]	28.7	25.3	28.0	29.3	0.55	*ns*
Albumins [mmol/l]	15.0	15.3	17.3	16.3	0.19	*ns*
Total cholesterol [mmol/l]	137	151	146	153	2.10	*ns*
Triglycerides[mmol/l]	33.7	25.1	20.2	34.0	1.54	*ns*
Calcium [mmol/l]	10.7	10.0	10.8	9.80	0.13	*ns*
Phosphorus [mmol/l]	7.22	7.36	7.32	7.79	0.12	*ns*
Glutathion [mmol/ml]	7.00^{A}	7.79^{A}	8.33^{A}	3.28^{B}	0.29	*< 0.001*
MDA [nM/ml]	1.14^{A}	1.32^{B}	1.57^{AB}	1.47^{B}	0.09	*0.001*

[†]500 HA-NP, eggs injected with 500 µg HA-NP/ml colloid; [‡]100 HA-NP, eggs injected with 100 µg HA-NP/ml colloid; [§]50 HA-NP, eggs injected with 50 µg HA-NP/ml colloid; [¶]C, control group, not injected; [#]SEM, standard error of the mean; *ns, non-significant; [+]AST, aspartate aminotransferase; [$]ALT, alanine aminotransferase; [‖]ALP, alkaline phosphatase; [◊]LDH, lactate dehydrogenase; [♦]MDA, malondialdehyde.
[A,B]Means not sharing the same superscript differ significantly at $p \leq 0.05$ ($n = 10$ per group).

Table 5. Concentration of selected oxidation markers in the breast muscle and liver of chicken embryos on d 20 after *in ovo* treatment of hydroxyapatite nanoparticles (HA-NP).

	Breast muscle				Liver			
	SOD[+] [U/mg]	GSH-PX[$] [IU/l]	TAS[‖] [µmol/mg]	GR[◊] [IU/l]	SOD [U/mg]	GSH-PX [IU/l]	TAS [µmol/mg]	GR [IU/l]
500 HA-NP[†]	0.60^{AB}	0.13	1.14^{B}	0.02	$<0.01^{B}$	0.11	0.28	0.01
100 HA-NP[‡]	0.17^{B}	0.11	0.61^{A}	<0.01	0.03^{A}	0.12	0.30	0.01
50 HA-NP[§]	0.31^{B}	0.14	0.71^{A}	0.01	0.09^{A}	0.11	0.33	0.02
C[¶]	0.87^{A}	0.12	0.69^{A}	0.02	0.02^{A}	0.13	0.33	0.01
SEM[#]	0.145	0.038	0.120	0.006	0.025	0.011	0.020	0.002
p-Value	*<0.001*	*ns**	*<0.001*	*ns*	*<0.05*	*ns*	*ns*	*ns*

[+]SOD, superoxide dismutase; [$]GSH-PX, glutathione peroxidase; [‖]TAS, total antioxidant status; [◊]GR, glutathione reductase; [†]500 HA-NP, eggs injected with 500 µg HA-NP/ml colloid; [‡]100 HA-NP, eggs injected with 100 µg HA-NP/ml colloid; [§]50 HA-NP, eggs injected with 50 µg HA-NP/ml colloid; [¶]C, control group, not injected; [#]SEM, standard error of the mean; *ns, non-significant.
[A,B]Means not sharing the same superscript differ significantly at $p \leq 0.05$ ($n = 10$ per group).

the liver, differences in SOD concentration were observed between the 500 HA-NP and all other groups. TAS concentration in the breast muscle was higher in 500 HA-NP than in the other treated groups. Generally, it was noted that TAS and SOD concentrations were higher in the breast muscle than in the liver. GSH-PX and RD concentrations were similar in the breast muscle and liver and not significantly different between treatments.

3.6. Bone turnover marker concentration

The concentrations of selected bone turnover markers measured in serum (Table 6) were not significantly different for BALP and OPG. PINP was not different, except 100 HA-NP

Table 6. The concentration of selected bone turnover markers in blood serum [ng/ml] of chicken embryos on d 20 after *in ovo* treatment with hydroxyapatite nanoparticles (HA-NP).

Group	BALP[+]	PINP[$]	OC[I]	OPG[◊]
500 HA-NP[†]	0.20	0.11[A]	12.3[A]	0.94
100 HA-NP[‡]	0.56	0.25[B]	4.6[B]	1.61
50 HA-NP[§]	0.65	0.07[A]	2.5[B]	0.99
C[¶]	0.57	0.06[A]	2.2[B]	1.00
SEM[#]	0.199	0.043	2.67	0.357
p-Value	*ns**	*0.05*	*<0.001*	*ns*

[+]BALP, bone-specific alkaline phosphatase; [$]PINP, procollagen Type I N-terminal propeptide; [I]OC, osteocalcin; [◊]OPG, osteoprotegerin; [†]500 HA-NP, eggs injected with 500 µg HA-NP/ml colloid; [‡]100 HA-NP, eggs injected with 100 µg HA-NP/ml colloid; [§]50 HA-NP, eggs injected with 50 µg HA-NP/ml colloid; [¶]C, control group, not injected; [#]SEM, standard error of the mean; *ns, non-significant.
[A,B]Means not sharing the same superscript differ significantly at $p \leq 0.05$ ($n = 10$ per group).

Table 7. The concentration of selected bone turnover markers in femur [pg/mg] of chicken embryos on d 20 after *in ovo* treatment with hydroxyapatite nanoparticles (HA-NP).

	PINP[$]	OC[I]
500 HA-NP[†]	0.27	28.2[C]
100 HA-NP[‡]	0.31	21.6[BC]
50 HA-NP[§]	0.25	18.2[AB]
C[¶]	0.31	13.1[A]
SEM[#]	0.067	3.84
p-Value	*ns**	*<0.05*

[$]PINP, procollagen Type I N-terminal propeptide; [I]OC, osteocalcin; [†]500 HA-NP, eggs injected with 500 µg HA-NP/ml colloid; [‡]100 HA-NP, eggs injected with 100 µg HA-NP/ml colloid; [§]50 HA-NP, eggs injected with 50 µg HA-NP/ml colloid; [¶]C, control group, not injected; [#]SEM, standard error of the mean; *ns, non-significant.
[A,B,C]Means not sharing the same superscript differ significantly at $p \leq 0.05$ ($n = 10$ per group).

level. Furthermore, per bone protein (Table 7) there were no significant differences in the concentration of PINP. Osteocalcin (OC) was the highest in serum and bone at the concentration of 500 HA-NP. Furthermore, in both serum and bone protein, OC concentrations showed a tendency to increase with the increasing level of HA-NP.

4. Discussion

The present study is one of the first to assess the effect of HA-NP injected *in ovo* on chicken embryo development, especially on the bone characteristics of the embryo.

It has to be noted that actually two control groups of eggs were measured, the group not injected and the group injected with ultra-pure water. However, for unknown reasons, the injected group had much higher mortality at the beginning of embryogenesis than other groups, and was excluded from statistical analysis. Consequently, the potential effects of the sham control-placebo mechanical manipulation could not be evaluated in the present work.

The hatchability and weight of the embryos indicated that IOI of HA-NP did not cause any negative effects on chicken embryo development, suggesting that it is a safe procedure. The body weight of the embryos was not affected by IOI of HA-NP, which is consistent with a recent study by Ahmadzadeh et al. (2019) using bacterial synthetised

ionic nano-hydroxyapatite and chemically synthesised hydroxyapatite applied *in ovo* which did not show any significant differences in hatchability and body mass of the chicken embryos. A previous study with calcium carbonate nanoparticles applied *in ovo* also did not show a negative effect on the hatchability of chickens (Salary et al. 2017).

In the present study, tibia weight was reduced in the 500 HA-NP group. It is difficult to make any clear explanation for the apparent reduction in bone weight, but the length, diameter, epiphysis diameter and the breaking strength were not significantly different. Furthermore, there were some significant differences in the bone weight, length and epiphysis diameter of femurs, but not in the bone diameter and breaking strength. It is well known that the bone development dynamics in general are different between tibia and femur, and these bones show different trends of changes in their mechanical, structural, and compositional properties during different periods of time (Yair et al. 2012). Oliveira et al. (2015) demonstrated no significant differences in tibia weight, length and width of broiler hatchlings after IOI of different organic microminerals: zinc, manganese and copper. Similar results were reported by Vijayakumar and Balakrishnan (2014), who demonstrated that calcium phosphate nanoparticles supplemented in a feed for broilers did not significantly influence the selected tibia bone parameters, for example, weight, length and width. However, the IOI of copper nanoparticles increased significantly femur weight and length in broilers (Mroczek-Sosnowska et al. 2017). Again, it is difficult to interpret unequivocally above results because of insufficient research on the effects of the IOI of HA-NP on bone parameters.

The concentration of ash in both types of bones was not affected. This result did not confirm results by Oliveira et al. (2015), where a high level of *in ovo* application of organic microminerals induced significantly higher content of bone ash. Furthermore, bone ash content is usually correlated with better mechanical properties (Yair et al. 2013), but this was not evaluated in the present study.

Ninety-nine per cent of Ca in the body is derived from the skeleton, where together with P forms hydroxyapatite. In the present study, no significant differences were observed in the content of Ca and P in both femur and tibia. This finding is not in agreement with the result of Oliveira et al. (2015) who studied *in ovo* application of different microelements on bone characteristics. It is worth noting that IOI to yolk sack of ionic nano-hydroxyapatite and chemically synthesised hydroxyapatite in concentrations of 50 and 100 μg/ml showed a higher potential than bacterial synthesised hydroxyapatite nanoparticles for the development of the chicken skeleton in comparison to chemically synthesised hydroxyapatite due to increasing bone mineral content and density (Ahmadzadeh et al. 2019).

The injection of carbonate nanoparticles significantly increased the content of Ca in tibia (Salary et al. 2017), while IOI of different nutrients increased Ca and P content in tibia on 19 d of incubation, but not on 21 d of incubation (Yair et al. 2013). Nevertheless, it should be noted that Ca and P concentrations in bones after *in ovo* enrichment depend on the type of nutrient injected (Yair and Uni 2011), and the place of injection. In this specific case HA-NP could not show any changes in bone mineralisation.

There is a possibility to determine the regularity or lack of selected physiological processes or disorders by selected biochemical parameters in the blood serum of birds (Schmidt et al. 2008). However, the biochemical parameters of the blood are rarely

specific markers of the organism's health, due to their conditioning by many factors (Krasnodębska-Depta and Koncicki 2000). Nevertheless, in the present study, no significant differences were observed, and the measured parameters were within the normal range for poultry. Furthermore, in the study of Ahmadzadeh et al. (2019), authors observed some toxic effects of chemically synthesised hydroxyapatite on the liver (higher values for AST, ALT) and kidney (higher values for urea nitrogen and creatinine) in comparison to bacterial synthesised hydroxyapatite nanoparticles; however, the statistical significance of these result was not provided.

During chicken embryo development, protection against peroxidative damage is provided by the combined action of a range of antioxidant components (Surai et al. 1996). An embryo relies on antioxidants accumulated in the egg yolk. Vitamin E or ascorbic acid plays a crucial role here. The first line of defence against reactive oxygen species (ROS) includes SOD, GSH-PX and GR (Fantel 1996; Surai et al. 1999). However, in the present experiment we did not note any changes in the activity of GSH-PX and GR. Despite the hatchability and weight of the embryos indicated that IOI of HA-NP did not cause any negative effects on chicken embryo development, the GSH data suggest that GSH synthesis followed the supplementation level of the HA-NP. Among the many enzymatic systems responsible for the intracellular redox balance maintenance, the main role of GSH is participation in the antioxidant defence system by neutralising ROS (Meister 1984; Wu et al. 2004). For some reason, the injected organism was in need to increase antioxidative capacity because of the circulation of HA-NP or/and the HA-NP may stimulate bone metabolism which also would increase cellular stress. However, the bone weight, in 500 HA-NP group, was negatively affected, thus this may reflect some toxic effects. It has been reported that chemical structure and the morphology of nanoparticles show key roles in the toxicity and their degradability (Ajita et al. 2015).

Moreover, during lipid peroxidation, MDA is produced which is commonly used as an indicator of lipid peroxidation in cells. In the present study, the results showed the lowest level of lipid peroxidation in the 500 HA-NP group. During normal chicken embryo development, there is a balance between antioxidation and pro-oxidation in tissues (Surai et al. 1999). In the present study, it was found that the SOD activity was higher in breast muscle than in the liver, thus suggesting its important antioxidant defence function in this tissue. This result is in agreement with the finding of Surai et al. (1999), who showed higher SOD activity in muscle than in the liver on 21 d of incubation. The TAS enables to assess the integrated antioxidant system that covers all biological components which exhibit antioxidant activity in a particular tissue. The results of the present study suggested that TAS was higher in the breast muscle than in the liver. A previous study has reported a negative correlation of TAS and MDA content in tissues, i.e. once the MDA content decreases, TAS increases (Kapusta et al. 2018). Although in the present study, the serum content of TAS was not determined, the highest TAS observed in the breast muscle in the 500 HA-NP group was concurrently characterised by the lowest MDA in serum. There is, however, still little understanding of the regulation of antioxidant systems in avian embryo development. Thus, further research on this topic is needed.

Bone modelling is defined as either the formation of bone by osteoblasts or resorption of bone by osteoclasts on a given surface. This contrasts with bone remodelling, in which

osteoblast and osteoclast activities occur sequentially in a coupled manner on a given bone surface (Allen and Burr 2014). The imbalance between these two processes might led to disease commonly known among elderly humans and in animals, which is characterised by loss of mineralised structural bones (Zhao et al. 2008; Vasikaran et al. 2011; Wheater et al. 2013). The bone modelling process is strictly associated with biochemical bone turnover markers, which play different roles in living organisms. OC is a major, non-collagenous protein in bones. This protein has been used as a biomarker of osteoblast activity for evaluating bone remodelling in humans and rodents (Kanbur et al. 2002; Seibel 2005). One form of OC known as carboxylated OC is incorporated into the bone matrix and is involved in bone mineralisation (Ducy et al. 1996). Few studies have investigated the use of OC as a bone turnover marker in birds. Jiang et al. (2013) demonstrated that OC is involved in bone remodelling in laying hens. They confirmed that OC concentration in serum of laying hens decreased with age.

The concentration of OC in serum was the highest in 500 HA-NP group. This value was much higher than results presented in the study of Jiang et al. (2013). However, the results from other groups were on a similar level in comparison with laying hens, which suggests that in general the OC concentration in serum is not considerably different in embryos and adult hens. Both Ca and P deficiencies reduce hydroxyapatite crystals formation. When the bone mineralisation decreases, free osteocalcin may be available for circulation in the blood (Iki et al. 2004). However, significantly higher OC levels in bone might indicate a higher mineralisation, but this could not be verified in the present study. The higher concentration of this biomarker in treatment groups (in serum and femur) could possibly suggest blocking bone mineralisation in response to the external source of hydroxyapatite. OC could be a preliminary biomarker of the early diagnosis of broiler osteoporosis; however, further research is recommended to determine the reference values of OC. BALP is another bone formation marker in humans and animals, including chickens (Seibel 2005). This enzyme is synthesised by osteoblasts and is assumed to be involved in the calcification of bone matrix, however, its precise role in the bone formation is still unknown. The elevated concentrations of ALP isoenzyme are generally attributed to either bone (BALP) or liver sources (more than 95%) (Masrour Roudsari and Mahjoub 2012). However, in the present study, we did not observe any significant differences for both ALP and BALP concentrations in serum.

Type I collagen, which constitutes 90% of bone proteins, is present mainly in connective tissue such as skin, tendons, and bones (Karim and Bhat 2009). Generally, the functions of collagen are directly related to cell growth, i.e. cell survival, proliferation and differentiation. It also helps in healing damaged bones and in maintaining structural integrity (Buehler 2006). Type I collagen is synthetised as type I procollagen, and during the extracellular processing of type I procollagen, there is cleavage of the amino terminal and carboxy terminal propeptides. Both propeptides are circulating in blood and are markers of bone formation. PINP is a useful indicator of bone diseases, such as in bone metastases of osteoblastic nature (Koivula et al. 2012). The current research commonly includes searching for new biomarkers of osteoporosis in humans, with the opportunity to use PINP. Kučukalić-Selimović et al. (2013) concluded that higher PINP level occurred in postmenopausal females with osteoporosis than in postmenopausal females with preserved bone mass. However, its low specificity does not warrant its usability in the diagnosis of osteoporosis. The present study showed similar PINP

concentrations in the serum and per bone protein of chicken embryo. This significant difference in PINP concentration in serum in 100 HA-NP group may indicate greater bone remodelling in this group. Nevertheless, it is not conclusive why the higher dosage of 500 HA-NP had no effect. There are no studies on PINP concentrations in chicken embryos or adult birds; hence, this subject area requires further research.

The last determined bone turnover biomarker in chicken embryo was OPG; it is a cytokine produced by osteoblasts, and its biological function is regulation of the differentiation and function of osteoclasts. Together with the receptor activator of nuclear factor ligand (RANKL), OPG inhibits osteoclast formation, function, and survival by preventing the binding of RANKL to the receptor activator of nuclear factor κB (RANK) (Lacey et al. 1998). RANK is found on dendritic cells, osteoclast precursors, and mature osteoclasts (Simonet et al. 1997). The discovery of the trio OPG, RANKL, and RANK has presented the opportunity to study the new area in the bone modelling process (Boyce and Xing 2008). The importance of OPG in osteoclastogenesis in avian species is still not well established. An *in vitro* study (Hou et al. 2011) showed that OPG inhibited osteoclast avian bone resorption and promoted osteoclast apoptosis; thus, it could be a target for regulating bone metabolism in chicken bone diseases. However, our results did not show any effect of HA-NP concentrations on the OPG level in serum. The present results are the first showing OPG in embryos, and more research on bone turnover markers during embryogenesis is needed. The fundamental fact is that each biomarker has its own specificity and its level depends on many other factors. With regard to the diagnosis of osteoporosis in birds, a reasonable necessity is to find proven and effective biomarkers which may indicate bone turnover during embryonic development.

It has to be noted that the present study is the first part of the project dealing with embryos. The second part focuses on the effects of *in ovo* injection during embryogenesis on post hatch growth and bone health. Considering that HA-NP did not show a positive effect at the embryo stage, they might affect performance and bone development in the post-hatching period. This will be determined in our follow-up studies.

5. Conclusions

The results of the present study indicate that *in ovo* application of HA-NP in the range of 50–500 μg/ml concentration of hydrocolloids did not affect hatchability and body weight of the embryos. Furthermore, in the group 500 HA-NP the weight of tibia and femur of the embryos was negatively affected. The content of Ca, P and ash was not affected by injection of HA-NP. The results also show modulatory effects of HA-NP on selected antioxidative markers. The treatment increased the necessity to detoxify ROS, which is evident from the highest GSH synthesis in 500 HA-NP group. The effect of HA-NP at the molecular level was observed in terms of their influence on bone turnover markers, especially OC of which the concentration was the highest in 500 HA-NP in serum and in femur. Thus, the *in ovo* application of HA-NP could modify the molecular responses at the stage of embryogenesis, but these changes were not reflected in embryo growth and even slowed down bone development. The question remains, whether *in ovo* administration of HA-NP would affect antioxidative status and bone turnover resulting in improved bone status and body gain in later life?

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Article

Calcium Carbonate Nanoparticles—Toxicity and Effect of In Ovo Inoculation on Chicken Embryo Development, Broiler Performance and Bone Status

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Simple Summary: Intensive selection in broiler chicken flocks has led do several leg disorders. The injection of nanoparticles, with high specificity to the bone, into the egg is a potential method to improve bone quality. The objective of our study was to evaluate the potential effect of calcium carbonate nanoparticles injected to the egg on chicken embryo development and bone quality of broiler chickens after 42 day of life. The calcium carbonate nanoparticles were not toxic to embryo and even improved the bone quality of embryos and later broilers without negative impact on production results. Thus, the application of calcium carbonate nanoparticles to the egg may be the potential solution for improving the bone mineralization of broiler chickens.

Abstract: The use of intensive selection procedure in modern broiler chicken lines has led to the development of several skeletal disorders in broiler chickens. Therefore, current research is focused on methods to improve the bone quality in birds. In ovo technology, using nanoparticles with a high specificity to bones, is a potential approach. The present study aimed to evaluate the effect of in ovo inoculation (IOI) of calcium carbonate nanoparticles (CCN) on chicken embryo development, health status, bone characteristics, and on broiler production results and bone quality. After assessing in vitro cell viability, the IOI procedure was performed with an injection of 500 µg/mL CCN. The control group was not inoculated with CCN. Hatchability, weight, and selected bone and serum parameters were measured in embryos. Part of hatchlings were reared under standard conditions until 42 days, and production results, meat quality, and bone quality of broilers were determined. CCN did not show cytotoxicity to cells and chicken embryo and positively influenced bone parameters of the embryos and of broilers later (calcification) without negatively affecting the production results. Thus, the IOI of CCN could modify the molecular responses at the stage of embryogenesis, resulting in better mineralization, and could provide a sustained effect, thereby improving bone quality in adult birds.

Keywords: calcium carbonate; nanoparticles; chicken embryo; broiler; bone quality

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1. Introduction

Currently, broiler lines are intensively selected for higher growth rate and increased final body weight (BW) [1]. However, this rapid weight gain in fast-growing chickens leads to several pathologies in the bones, such as osteoporosis and deformations in the legs [2]. This results in high economic losses, because skeletal abnormalities are one of the most common problems in modern chicken industries and cause reduction in consumed meat quality [3]. Moreover, broken legs lead to decreased feed intake, which eventually results in reduced final BW [4]. Bone strength in broiler legs is strictly associated with mineralization, especially Ca deposition, which is 99% localized in the bones [5,6]. This macroelement contributes to the mineral structure of the bone with P to form hydroxyapatite. In addition, Ca widely contributes to many essential physiological processes such as cell proliferation, muscle contraction, blood coagulation, enzyme activation, and hormone secretion [6,7]. Among all the Ca sources, limestone, a naturally occurring mineral primarily composed of calcium carbonate ($CaCO_3$), is the predominant Ca supplement used in broiler production [8]. The inorganic forms of elements, such as oxides, sulfates, and carbonates, are widely used in poultry nutrition, mainly for economic reasons [9]. The concentration and bioavailability of Ca can differ according to the source or particle size [8,10]. Currently, there is a growing interest in the application of nanotechnology to produce a supplemental source of minerals in poultry diets. The use of nanoparticles as sources of various elements in poultry nutrition is a response to today's consumption trends and society expectations. Minerals administered in the nanometric size can improve the welfare and health of animals because of their better availability [11]. Moreover, the aspect of environmental protection is also important—nanoparticles, as an innovative alternative to conventional sources of minerals, can be better absorbed by animals, thus reducing the excretion of minerals [12–14]. Calcium compounds in nanoparticle forms have a high prospective in poultry production [15]; so far, however, research is limited. Most studies are primarily concerned with per os administration of Ca nanoparticles [13,16–18]. The supplement of calcium compounds in the form of nanoparticles is also considered as a strategy to bring down the cost of calcium (and phosphorus) supplement in the feed [16]. The IOI of nutrients remains an interesting, alternative method of providing functional nutrition using various compounds. Nutrient supplementation by IOI could be more efficient when a selected compound is attached to nanoparticles, because of their ability to effectively deliver the compound inside cells and body tissues due to their small size (1–100 nm) [19]. According to Sawosz et al. and Zielinska et al. [20,21], the administration of nanoparticles directly to the egg at the early stage of embryogenesis can lead to a number of molecular and systemic changes. This, in turn, can enable a "better start" for newly hatched chickens and then influence the health and production status of the birds at later stages of life. For example, Mroczek-Sosnowska et al. [22] suggested that Cu nanoparticles administered in ovo interfere with the molecular status of muscle maturation during embryogenesis via MyoD1 and Pax7 proteins and later actually proved that breast muscle was bigger in broilers [23]. In line with this approach, we hypothesized that calcium carbonate ($CaCO_3$) nanoparticles (CCN) as a safe and nontoxic supplement can be delivered by IOI at the early stage of incubation. They can exert several stimulative and modulatory effects on the development of the chicken embryo and then influence the bone quality of broiler without affecting production results. CCN as an external source of Ca may affect the regulation of bone osteocalcin (OC), the protein responsible for hydroxyapatite binding, ultimately resulting in increasing bone mineralization. Studies focusing on IOI of calcium compound nanoparticles are very limited [24–26]. The present study aimed to evaluate the effect of the IOI of CCN on chicken embryo development, health status, bone characteristics, and production results as well as meat quality and bone quality of broiler chickens after 42 days of rearing.

2. Materials and Methods

2.1. Nanoparticle Characterization and Preparation

CCN (white nanopowder, 97.5%) were obtained from SkySpring Nanomaterials, Inc. (Houston, TX, USA). Hydrocolloids of CCN of increasing concentrations (5, 10, 25, 50, 100, and 500 µg/mL) were produced by mixing the nanopowder with ultrapure water. After premixing the whole solution, ultrasound was introduced into the solution for 45 min by using Ultron U-509 apparatus (Transfer Multisort Elektronik, Lodz, Poland). The average zeta potential and particle size determination was carried out using Zetasizer Nano-ZS ZEN 3600 (Malvern Instruments Ltd., Malvern, UK) with the dynamic light scattering mode and laser Doppler electrophoresis at room temperature (23 °C). The size and shape of a single CCN were visualized using a Morgagni 268D transmission electron microscope with a wide-angle Olympus Morada digital camera (FEI, Hillsboro, OR, USA). The individual CCN hydrocolloids were prepared 30 min before the specific procedure was performed in each part of the experiment.

2.2. In Vitro Cytotoxicity and Mineralization

2.2.1. Cell Isolation

Fertilized chicken eggs (*Gallus gallus domesticus*; $n = 20$) were supplied by a commercial, certified hatchery. On the twelfth day of incubation, the embryos were sacrificed, and their femurs were collected to obtain the primary osteogenic cells using modified method for bone cells isolation described by Li et al. [27]. The bones were cleaned of soft tissues; the bone shaft was isolated, crushed mechanically, and treated with collagenase for 30 min. The suspension was filtered through a 74-µm mesh sieve and centrifuged at 200× *g* for 10 min at room temperature. The supernatant was discarded, and the pellet was resuspended in medium containing DMEM and 10% (v/v) PBS.

2.2.2. Viability Assay

Cell viability was evaluated using a 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxyanilide salt (XTT)-based cell proliferation assay kit (Life Technologies, Taastrup, Denmark). Cells were plated in 96-well plates (5×10^3 cells per well) and incubated for 24 h. The medium was then removed, and CNN samples at concentrations of 5, 10, 25, 50, and 100 µg/mL were introduced into the medium. Next, 50 µL of XTT solution was added to each well and incubated for an additional 3 h at 37 °C. The optical density of each well was recorded at 450 nm by using an enzyme-linked immunosorbent assay reader (Infinite M200, Tecan, Durham, NC, USA). Cell viability was expressed as a percentage $(OD_{test} - OD_{blank})/(OD_{control} - OD_{blank}) \times 100$, where OD_{test} is the optical density of cells exposed to CCN, $OD_{control}$ is the optical density of the control sample, and OD_{blank} is the optical density of wells without cells.

2.2.3. Cell Staining

Primary osteogenic cells were seeded in six-well plates (1×10^5 cells per well) and incubated for 24 h. Cells cultured in the medium without the addition of CCN were used as the control. CCN were added to the cells at increasing concentrations (5, 10, 20, 50, and 100 mL/L). After 24 h, the cells were fixed with 4% paraformaldehyde and stained with a 2% Alizarin red solution (Merck, Warsaw, Poland) [28]. Cell morphology was recorded using an optical microscope (TL-LED, Leica Microsystems, Wetzlar, Germany).

2.3. In Ovo Experimental Design

According to 3rd Local Ethics Committee for Animal Experiments in Warsaw University of Life Sciences, the experiments on chicken embryo and broilers followed the approval of Local Ethics Committee (Approval No. 46/2015). The experimental material consisted of 240 fertilized chicken eggs from 37-week-old Ross × Ross 308 hens. First, the eggs were stored in a refrigerator at 12 °C and 73% humidity for 2 days and then placed in an incubator (Jamesway, Cambridge, ON, Canada). The eggs were weighed (55.9 ± 1.83

g) on day 1 of incubation and randomly divided into two groups, with 120 eggs per group. The control group was not inoculated, and the experimental group was supplemented with 500 µg/mL hydrocolloid of CCN in 0.2 mL volume per egg. Negative control (inoculated with PBS) was not included in this study. Before the injection procedure, the eggs were immersed in a 0.5% solution of potassium permanganate. The hydrocolloid was inoculated on the first day of incubation under sterile conditions into the albumen, using 27-gauge, 20-mm needles. The hole was sealed using a sterile tape, and the eggs were placed in an incubator immediately after the injection. Standard incubation conditions were provided to all eggs (temperature 37.8 °C, humidity 55%, turned once per hour for the first 18 days; 37 °C and 75% humidity on days 19 and 20). During incubation, the eggs were candled on the 7th and 18th d to discard unfertilized eggs and determine the dead embryo percentage. The hatchability was calculated as the ratio of eggs with living embryos on day 20 to the number of fertilized eggs in each group.

On day 20, eggs from each group were randomly selected, and all embryos were weighted and decapitated. During decapitation, pooled blood from 1.5 embryos (about 1.5 mL per sample) was collected (n = 10) from the jugular artery into glass tubes, stored at 4 °C overnight, and then centrifuged for 5 min at 1200× g (MPW-350R centrifuge, MPW Med. Instruments, Poland). The obtained serum was stored in cryovials at −80 °C. Previously selected embryos (10 per group) were immediately transferred on dry ice after blood collection. After cooling, selected organs were collected (liver, muscle, and both femurs and tibias), weighed, and stored at −80 °C. IOI procedure and the minimum necessary sample size estimation were performed with minor modifications as described by Lukasiewicz et al. and Pineda et al. [29,30].

2.4. Embryo Serum Biochemical and Toxicity Analyses

Ten serum samples from each group were analyzed by standard laboratory procedures in the Veterinary Diagnostic Laboratory at Warsaw University of Life Sciences by using commercial kits. Selected biochemical kits were used to detect the level of liver enzymes, including aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), and lactate dehydrogenase (LDH), as well as kidney-related biochemical factors such as calcium, phosphorus, and creatinine. The levels of glucose (Glu), total protein (TP), albumin (Alb), total cholesterol (TC), and triglyceride (TG) were determined. Glutathione (GSH) was measured quantitively using Ellman's method modified by Matusiewicz et al. [31]; in this method, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB, Ellman's reagent) is reduced by thiol compounds to form a colored product (2-nitro-5-mercaptobenzoic acid) with the maximum absorbance at 412 nm. To 375 µL of serum samples from each group, 19.7 µL of 50% trichloroacetic acid (TCA) was added and centrifuged (1200× g, 5 min). Then, 6.25 µl of deproteinized supernatants was transferred into a microplate and mixed with 50 µL of 0.2 M phosphate buffer (PBS) and 6.25 µL of 6×10^{-3} M DTNB. The absorbance was measured using Tecan's NanoQuant Infinite M200 PRO (Tecan Austria GmBH, Grödig, Austria) analyzer.

Ten serum samples of 250 µL were used to determine malondialdehyde (MDA) level according to the method proposed by Kapusta et al. [32]. First, 25 µL of 0.2% 2,6-bis(1,1-dimethylo)-4-methylphenol (BHT, in ethanol) and 1 mL of 5% trichloroacetic acid (aqueous, TCA, Merck, Warsaw, Poland) were added to each sample and vortexed. After centrifugation at 14,000× g for 10 min, 750 µL of supernatant was transferred to a glass tube, and 500 µL of 0.6% thiobarbituric acid (aqueous, Merck, Warsaw, Poland) was added, mixed, and incubated for 45 min in a water bath at 90 °C. The supernatants were then stored in cool conditions and centrifuged at 4000× g for 5 min. Then, 100 µL of clear supernatant was transferred into a microplate. MDA concentration was determined using Tecan's NanoQuant Infinite M200 PRO analyzer at the wavelength of 532 nm.

2.5. Embryo Bone Measurements

All collected tibias and femurs were cleaned, and their length and diameter in the middle of the shaft were measured using an electronic caliper for linear measurements. The breaking resistance of bones was then determined using a Zwick Testing Machine (Z0.5 Zwicki-Line, Ulm, Germany) with a warhead equipped with a Warner-Bratzel blade with a maximum force of 1 kN. The blade movement speed was 50 mm/min. The six femurs and tibias were weighed on an analytical balance in quartz beakers (weighing approximately 0.1 g), smoked on a hot plate (max. temp. 400 °C), and burned in a muffle furnace with temperature control at approximately 470 °C for 36 h. Subsequently, cooling was performed by adding 2 mL of redistilled water and HCl (38%) to the individual samples. The samples were transferred quantitatively with 40 mL of redistilled water. The mineral content of Ca and P was determined using an ICP-AES Thermo iCAP 6500 DUO (Thermo Fisher Scientific, Waltham, MA, USA) atomic emission spectrometer. Six left femurs were initially grounded in mortar on ice, suspended in 1 mL of PBS, and homogenized. The protein level in sample homogenates was determined using the Bicinchoninic Acid Kit (BCA, Merck, Warsaw, Poland). Then, 25 µL of homogenized supernatants was transferred into a microplate in two replicates, and 200 µL of the reagent mixture was added. The analyzer wavelength was 562 nm. The concentration of OC in homogenates was determined using a chicken-specific enzyme-linked immunosorbent assay (ELISA) kit obtained from Immunogen, Warsaw, Poland (catalog no. MBS268643) and measured using Tecan's NanoQuant Infinite M200 PRO analyzer at 450 nm according to the protocol. OC and bone alkaline phosphatase (BALP, catalog no. MBS2512530) were also determined in serum.

2.6. Broiler Chicken Management

After hatching from eggs previously inoculated with CCN and noninjected ones, 1-day-old chicks from each group were randomly selected for further rearing (90 chicks per group) and not sorted by sex. The next part of the experiment was conducted at the Agricultural Experimental Farm, Wilanów-Obory. The chicks were vaccinated against Marek, Gumboro, and infectious diseases and randomly divided into three replicates of 30 chicks per replicate and placed in rearing boxes with proper dimensions for stocking density. On the 15th day of rearing, vaccination against Gumboro and infectious bronchitis was repeated. The birds were kept on chopped straw litter under standard conditions: The temperature was 32 °C in the first week and lowered by 2 °C weekly to 20 °C in the last week. The humidity was 60%, and 24-h lighting was applied. Microclimate parameters such as humidity and toxic gas content (ammonia, carbon dioxide, and hydrogen sulfide) were recorded at weekly intervals. All parameters were below the standards established by the Regulation of the Minister of Agriculture and Rural Development of 15 February 2010 (Journal of Laws No. 56, item 344) [33]. The birds had free access to water and were fed ad libitum. The applied feed mixtures were starter for chickens at 1–10 days, grower at 11–34 days, and finisher at 35–42 days, formulated in accordance with Ross 308 Nutrition Specifications [34] (Table 1). The BW was recorded at 1, 10, 35, and 42 days of rearing. The feed intake and mortality were registered daily, and final mortality (%) and feed conversion ratio (kg/kg). The mortality, determined by dividing number of dead birds in each group by the initial number of birds in the group and multiplied by 100, FCR = total feed intake/total final BW.

At the end of the experiment, six cocks from each group, having BW within the average BW of the group, were selected, and transferred to a separate slaughterhouse, and euthanasia was performed by decapitation. According to 3rd Local Ethics Committee for Animal Experiments in Warsaw University of Life Sciences, the experiments on chicken embryo and broilers followed the approval of Local Ethics Committee (Approval No. 46/2015). The twelve carcasses were chilled by blowing at 4 °C for 24 h. The cooled carcasses were weighed and then dissected. The contents of breast muscles, leg muscles, heart, liver, and gizzard were determined in relation to BW before slaughter. During decapitation, blood collection was performed.

Table 1. Components and chemical composition of the broiler chicken diets.

Ingredients, g/kg	Starter (Days 1–10)	Grower (Days 11–34)	Finisher (Days 35–42)
Maize	100	114	100
Wheat	530	550	608
Extracted soybean meal	306	274	216
Calcium	11.9	12.0	9.7
Sodium bicarbonate	2.0	1.4	1.6
NaCl	2.4	2.8	2.6
Stimulator	0.1	0.1	0.1
Dicalcium phosphate	11.8	7.8	6.4
Oil	21.0	24.0	44.0
Methionine 84%	4.8	4.2	2.8
Lysine	3.6	3.4	2.8
Threonine	1.4	1.3	1.0
Premix *	5.0	5.0	5.0
Nutrient Composition, g/kg			
Analyzed			
Crude protein	219	207	187
Crude fat	47.1	52.3	68.1
Ash	50.7	51.3	51.7
Calculated			
Lysine	12.8	11.8	11.1
Methionine	7.2	6.3	5.4
Calcium	9.5	7.0	7.5
Phosphorus	6.6	5.3	5.1
Metabolisable energy (MJ/kg)	12.28	12.54	12.75

* Rovimix (DSM, Poland): A (retinol acetate) 2,200,000 IU/kg, D3 (E671) 500,000 IU/kg, E (di-alpha-tocopherol acetate 10,000 mg/kg, D (D-pantothenate calcium) 2722 mg/kg, K3 (MNB) 500 mg/kg, B1 (thiamine mononitrate) 400 mg/kg B2 (riboflavin) 1400 mg/kg, B6 (pyridoxine hydrochloride) 800 mg/kg, B12 (cyanocolbalamin) 400 µg/kg, niacin (nicotinic acid) 8000 mg/kg, folic acid 200 mg/kg, biotin 30,000 µg/kg, choline chloride 60,000 mg/kg, copper 1500 mg/kg, zinc (zinc oxide) 11,000 mg/kg, manganese 14,000 mg/kg, iodine (calcium iodate) 120 mg/kg, selenium (sodium selenate) 70 mg/kg, iron (iron sulphate) 9000 mg/kg, citric acid 19 mg/kg, etoxyquin 34.8 mg/kg, propyl gallate 5.4 mg/kg, calcium carbonate 251 g/kg, magnesium 2.2 g/kg.

2.7. Meat Quality and Bone Analyses

The pH of breast muscle was measured 24 h after slaughter according to PN-ISO 2917 by using the CP-411 pH meter (Elmetron, Zabrze, Poland) with a combined glass and calomel electrode. The device was previously calibrated in the presence of buffers at pH 4.0 and 7.0. Color parameters were determined for shredded breast muscle using the CR-410 colorimeter (Minolta Co. Ltd., Osaka, Japan) according to the manufacturer's protocol. Each measurement was performed in two replicates. The values for parameter L* (brightness) were obtained from 0 to 100. Parameters a* and b* are coordinates of trichromaticity. The value +a* corresponds to red, −a* to green, +b* to yellow, and −b* to blue.

The collected and cleaned femurs (right and left) were measured (weight and length), and left bones were prepared for determining breaking resistance using the same method as described above (cf: embryo bone measurements). Subsequently, 3 g of bone (proximal metaphysis and epiphysis area) were obtained by initially grounding in mortar on ice. Ca and P were then evaluated in the fragmented tissue. The concentrations of microminerals (Mg, Mn, Zn, and Cu) were determined using ICP-AES Thermo iCAP 6500 DUO (Thermo Fisher Scientific, Waltham, MA, USA) atomic emission spectrometer was performed. Next, 0.2 g of fragmented femur was lyophilized for 24 h suspended in 1 mL of RIPA buffer (Merck, Warsaw, Poland) for 5 days and homogenized. OC and protein concentration in the homogenates were evaluated (cf: embryo bone measurements).

Right femurs were scanned using a multi-slice 64-row CT scanner (750 Revolution CT, GE Healthcare, Waukesha, WI, USA) following the Gemstone Spectral Imaging (GSI) protocol. The following parameters were used: amperage: ~260 mA; rotation: 0.08/s;

HE+: 19.4 mm/rot; slice thickness: 0.6 mm; voltage: GSI-QC (Dual Energy). Images were analyzed with AW VolumeShare7 software (GE Healthcare, Waukesha, WI, USA) in the bone window (W = 2000; L = 350). The auto contour measuring tool was used to fit the measuring window to the bone size in the sagittal, coronal, and axial planes. The current threshold was set at 42 to measure the average bone volume [cm^3] and the average relative bone density in Hounsfield Units [HU] separately for each bone. The threshold was then adjusted separately for each bone to achieve the average bone volume [cm^3] for relative bone density of 500 [HU] and the average bone volume [cm^3] for relative bone density of 1000 [HU].

From the right femur in the area of proximal metaphysis, 0.5-cm-thick fragments were cut, immersed in 10% neutral formalin for 72 h, and decalcified in 15% neutral EDTA buffer (pH = 7.4) (Merck, Warsaw, Poland) for 1 month. The decalcified femurs were dehydrated with graded ethanol (5–100%), defatted in xylene, and embedded in paraffin. Sections of approximately 6 µm thickness were prepared using Microtome Leica RM 2265 (Leica Biosystems, Nussloch, Germany) and used for hematoxylin and eosin (HE) and alizarin red staining. Staining with alizarin red S (Merck, Warsaw, Poland) was performed as follows. First, the rehydrated and defatted sections were stained with 2% alizarin red S solution for 2 min. The sections were rapidly dipped into acetone and acetone xylene (50/50) for 2 s [35]. Microscopic visualization was acquired using a Nikon Eclipse 90i light microscope with a Nikon Digital Sight DS-U1 camera (Nikon Corporation, Tokyo, Japan) and NIS-Elements "D" (Documentation) v.5.02.03 software (Nikon Corporation, Tokyo, Japan). Three visualizations were acquired from each section (18 in total per group) from the repetitive area. The average intensities of alizarin red S staining were quantified using NIS-Elements "D" (Documentation) v.5.02.03 software [36].

2.8. Statistical Analysis

The collected data were subjected to statistical analysis using the general linear model of one-factor analysis of variance (ANOVA) with IBM SPSS Statistics v.21.0. The level of significance was set at $p \leq 0.05$. Individual embryos and birds were treated as experimental units in accordance with the principles of minimum necessary sample size.

3. Results

3.1. Physicochemical Properties of CCN

The zeta potential of CCN was approximately 20 mV, indicating relative stability. The nanoparticles showed cubic shape with an average size of 15–40 nm. The average size of agglomerate was over 1000 nm (Figure 1).

3.2. In Vitro Toxicity and Nineralization Results

Figure 2 shows the results of cytotoxicity test (XTT) of CCN in the concentration range of 5 to 100 µg/mL. The viability of cells was not affected negatively by CCN (no toxic effect). Moreover, the viability of the cells was even higher at higher concentrations of nanoparticles, thus suggesting stimulative effect of CCN on cell viability (high osteoconductive properties). The calcified nodules appeared bright red with higher intensity and density following the increase in the concentration of CCN added to cells, which suggest more effective mineralization processes in these cultures.

3.3. In Ovo Results

The results indicated that in IOI of CCN at the concentration of 500 µg/mL did not negatively affect hatchability of the embryos. The hatchability level in both groups was high (over 90%), and mortality caused by infection was negligible. No defects were observed in the developed embryos. The BW of the embryo and breast muscle weight were not affected by CCN. However, the liver weight was affected and was higher in the CCN group ($p \leq 0.05$) (Figure 3).

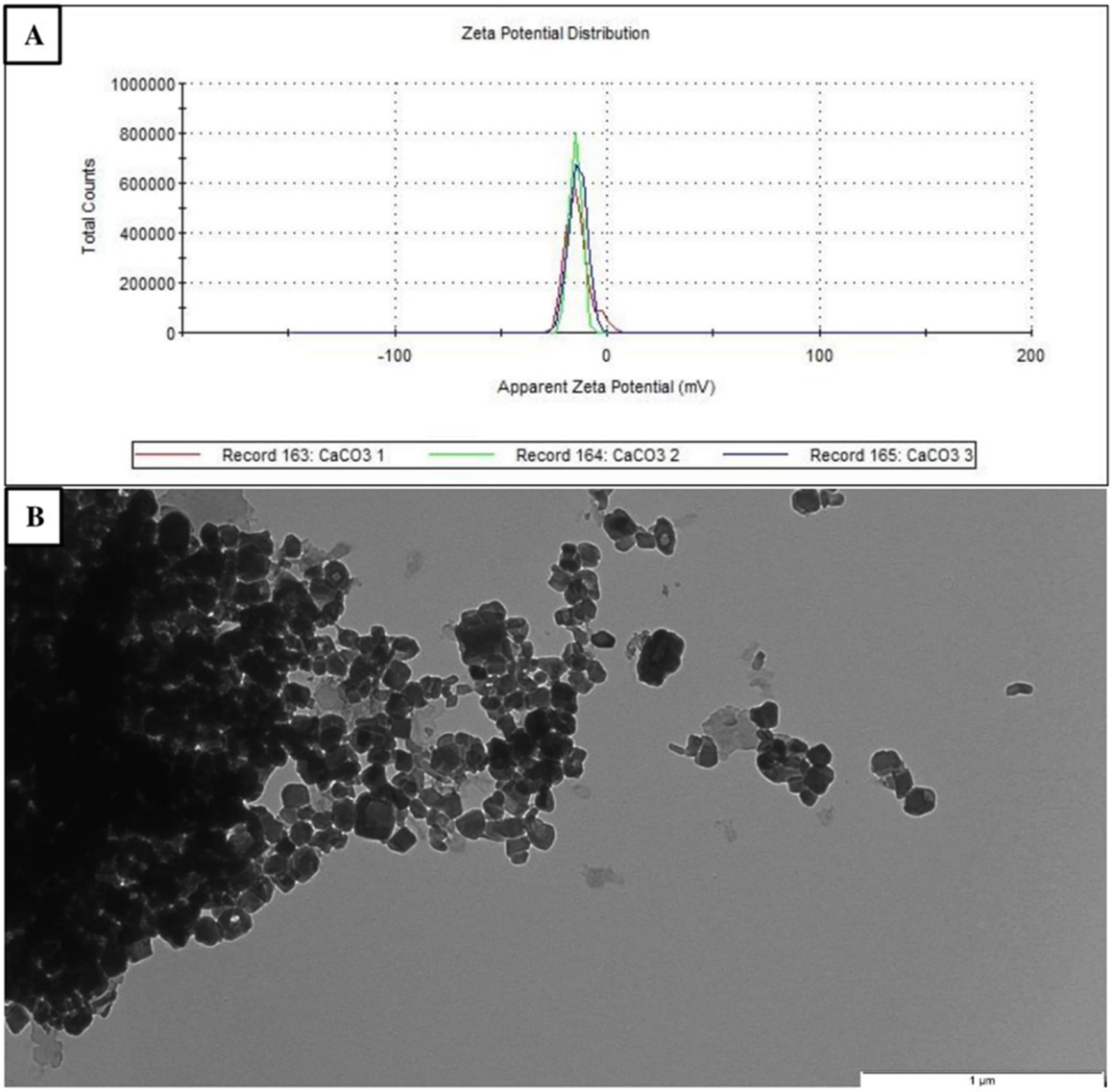

Figure 1. Representative zeta potential of CCN at the concentration of 50 µg/mL (three peaks) (**A**). Transmission electron microscope image of CCN. Scale bar represents 1 µm (**B**).

The serologic parameters of chicken embryos (Table 2), in most cases, showed no significant differences between the groups, thus indicating normal liver and kidney function. However, MDA concentration was significantly decreased in the CCN group ($p \leq 0.05$), which may suggest higher peroxidation of lipids. Moreover, the glucose (Glu) concentration was significantly reduced in CCN group.

Figure 4 shows the results of selected measurements performed in the femur and tibia of embryos. No significant differences in bone length, diameter, and breaking resistance were observed between the groups; however, the tibia weight was higher ($p \leq 0.05$) in the CCN group. The Ca and P concentrations in the femur and tibia were higher in the CCN group ($p \leq 0.05$), indicating better mineralization in bones of embryos from the IOI group.

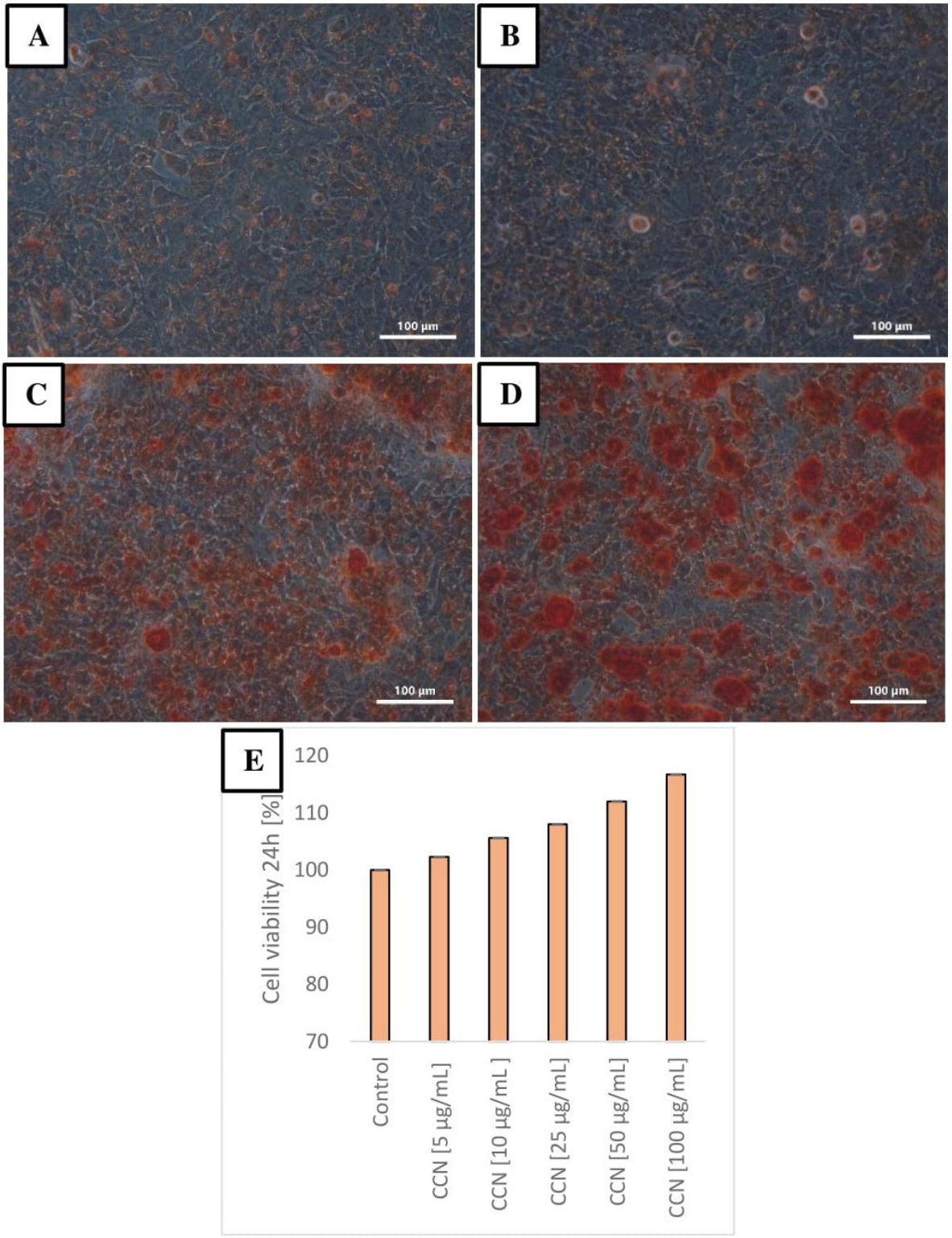

Figure 2. Alizarin red staining for mineralization. The calcified nodules appeared bright red color (original magnification ×100). Cells in control group, without CNN (**A**). Cells incubated with CCN at 5 µg/mL (**B**). Cells incubated with CCN at 25 µg/mL (**C**). Cells incubated with CNN at 100 µg/mL (**D**). Cell viability in groups with increasing CCN concentration determined by the XTT assay after 24 h of incubation (**E**). The error lines represent standard error of mean.

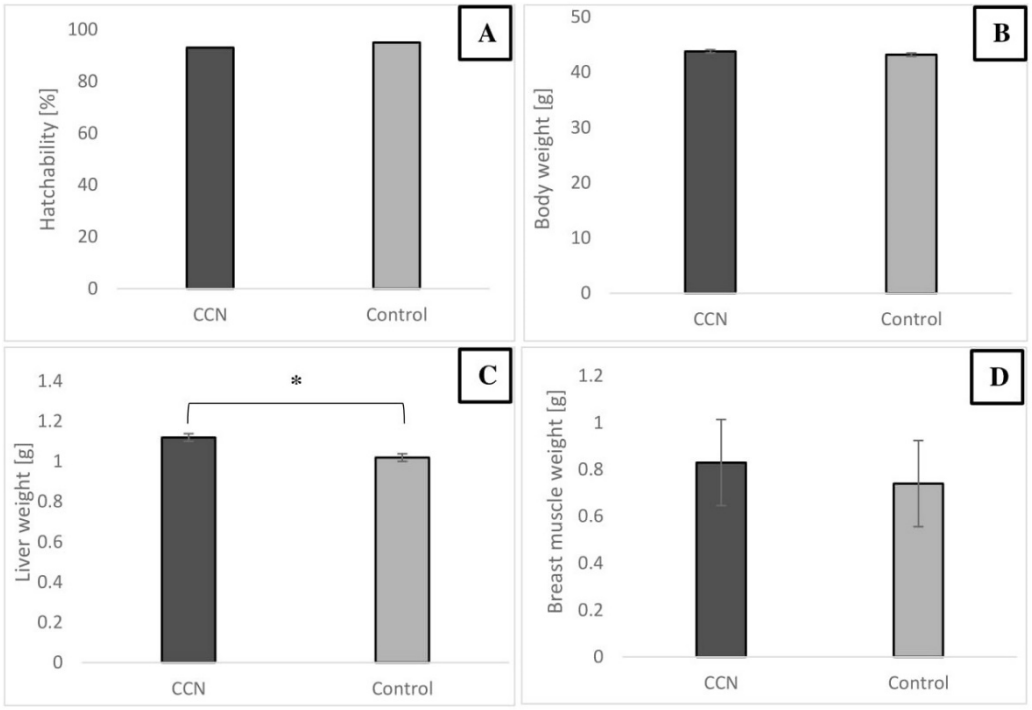

Figure 3. Hatchability (**A**), BW (**B**), liver weight (**C**), breast muscle weight (**D**) of chicken embryos on day 20 after IOI with 500 μg/mL of CCN. * Value on bars differs significantly at $p \leq 0.05$. The error lines represent standard error of mean.

Table 2. Serum parameters of chicken embryos.

Parameter	Group		SEM	*p*-Value
	CCN	Control		
AST [U/L]	256	240	31.71	0.716
ALT [U/L]	10.0	6.93	1.774	0.227
ALP [U/L]	948	837	73.43	0.209
BALP [ng/mL]	0.40	0.57	0.053	0.336
Alb [mmol/mL]	15.0	15.3	0.491	0.330
TP [mmol/mL]	24.7	26.0	1.465	0.054
Glu [mmol/mL]	259	276	5.283	0.028
TC [mmol/mL]	139	127	5.564	0.104
TG [mmol/mL]	28.2	32.7	4.066	0.432
LDH [U/L]	1120	1052	98.66	0.413
Cr [mmol/mL]	0.33	0.41	0.022	0.131
MDA [nM/mL]	1.33	1.47	0.022	0.000
GSH [mmol/mL]	3.65	3.30	0.673	0.248
Ca [mmol/mL]	10.5	10.1	0.347	0.248
P [mmol/mL]	7.01	7.58	0.307	0.623

SEM: standard error of mean; CCN: embryos from eggs inoculated with 500 ug/mL hydrocolloid of calcium carbonate nanoparticles; AST: aspartate aminotransferase; ALT: alanine aminotransferase; ALP: alkaline phosphatase; BALP: bone alkaline phosphatase; LDH: lactate dehydrogenase; Glu: glucose; Cr: creatinine; TP: total protein; Alb: albumins; TC: total cholesterol; TG: triglycerides; GSH: glutathione; MDA: malondialdehyde.

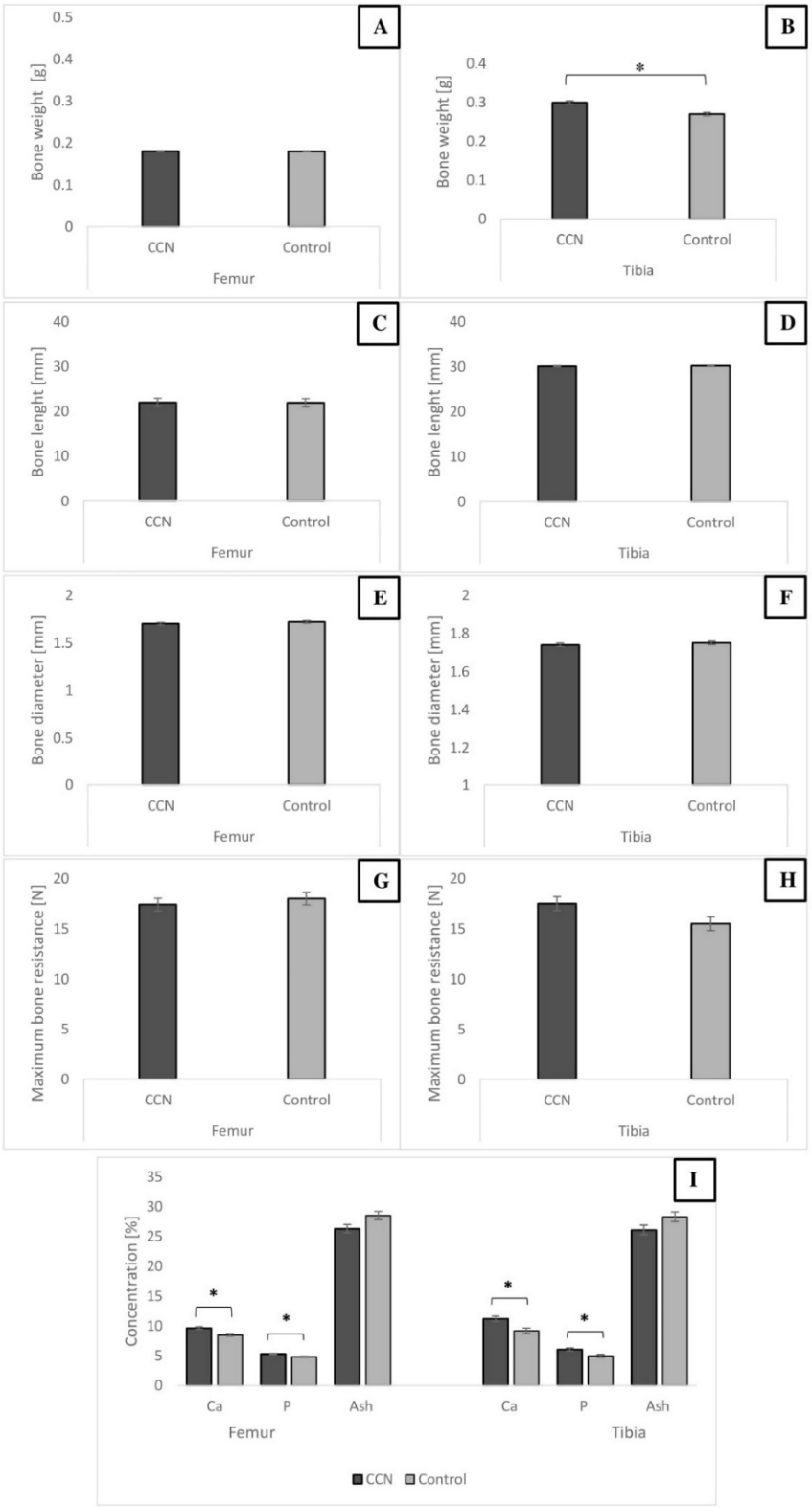

Figure 4. Selected parameters of chicken embryo bones on 20 day after IOI with CNN. Femur and tibia weight (**A**,**B**), femur and tibia length (**C**,**D**), femur and tibia middle diaphysis diameter (**E**,**F**), femur and tibia maximum resistance to breaking (**G**,**H**), femur and tibia Ca, P and ash content (**I**). * Value on bars differs significantly at $p \leq 0.05$. The error lines represent standard error of mean.

3.4. Production Results and Meat Quality of Broiler Chickens

The IOI with 500 µg/mL concentration of CCN did not negatively influence the production results and health of the birds from day 1 to day 42 of rearing. The initial BW of birds was similar. There were no differences in the BW on days 1, 10, 35 and 42 (43.3 g vs. 44.1 g, 278.6 g vs. 286.8 g, 2577 g vs. 2558 g, and 3134 g vs. 3147 g for CCN group compared to the control group). The feed conversion rate (FCR) also did not differ significantly between the groups (1.59 vs. 1.54 for the CCN and control groups, respectively). The mortality was at the acceptable level in both groups (Figure 5). The production results were in line with Aviagen recommendations and BW was even higher compared to Ross 308 performance objectives [37].

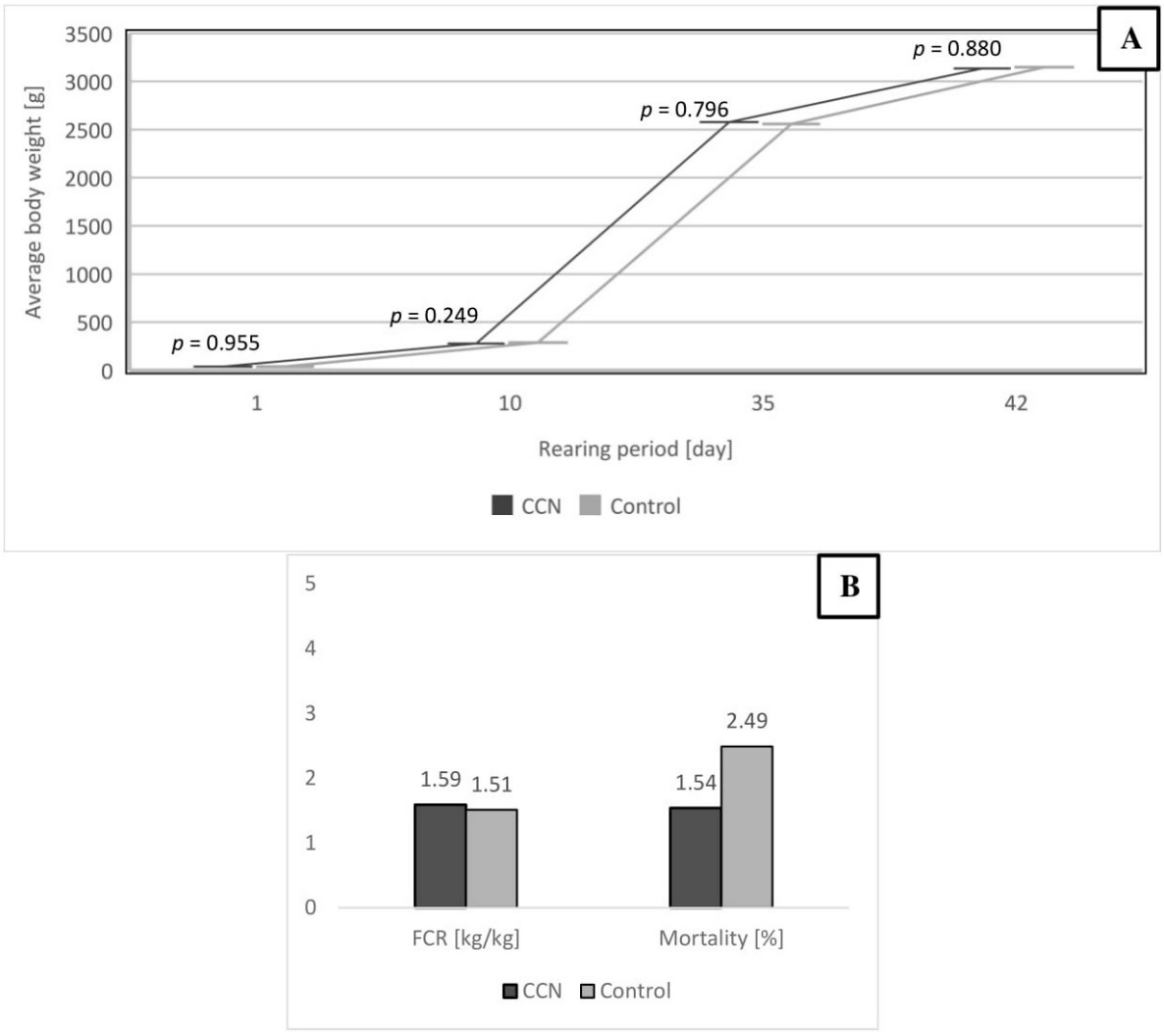

Figure 5. Production results of broiler chickens after 42 d of rearing. Average BW in groups on days 1, 10, 35 and 42. SEM values for average BW on days 1, 10, 35, and 42: 0.239, 3.496, 35.48, and 44.49, respectively (**A**). FCR and mortality of broiler chickens after the rearing period (**B**).

Table 3 shows the slaughter analysis of male carcasses, where not significant different between the groups. The pH of breast muscles was not significantly different; similar findings were noted for color parameters, except parameter a*. Table 4 shows selected physicochemical properties of breast muscle of broiler chickens.

Table 3. Results of male broiler chickens slaughter analysis.

Parameter	Group		SEM	*p*-Value
	CCN	Control		
BW before slaughter [g]	3499	3507	20.21	0.848
Dressing percentage [%]	78.6	78.4	0.207	0.689
Breast muscles [g/100 g BW]	29.2	30.4	0.418	0.136
Leg muscles [g/100 g BW]	19.4	19.3	0.373	0.166
Gizzard [g/100 g BW]	0.82	0.73	0.029	0.102
Liver [g/100 g BW]	2.34	2.12	0.073	0.134
Heart [g/100 g BW]	0.74	0.77	0.035	0.719
Total offal [g/100 g BW]	3.91	3.62	0.080	0.069
Fat [g/100 g BW]	1.64	1.40	0.086	0.181

CCN: embryos from eggs inoculated with 500 ug/mL hydrocolloid of calcium carbonate nanoparticles.

Table 4. pH and color parameters of breast muscle of male broiler chickens.

Parameter	Group		SEM	*p*-Value
	CCN	Control		
pH	5.78	5.83	0.015	0.128
L*	66.9	64.3	1.151	0.267
a*	14.8	16.4	0.289	0.002
b*	13.5	12.2	0.482	0.198

SEM: standard error of mean; CCN: embryos from eggs inoculated with 500 ug/mL hydrocolloid of calcium carbonate nanoparticles; System L*a*b*, where the L* value designates lightness, ranging from 0 for black to 100 for ideal white, whereas a* and b* are colour coordinates (+a* = redness, –a* = green, +b* = yellow, –b* = blue).

Bone Characteristics of Broiler Chickens

Ca concentration in the femur bone of broiler chickens was significantly higher in the CCN group than in the control group ($p \leq 0.05$). The relative bone density in Hounsfield scale tended to be higher in the CCN group ($p = 0.053$). The study did not show any significant differences in bone weight, length, and micromineral (Mg, Mn, Zn, and Cu) content (Figure 6).

Figure 7 shows the optical micrographs of the cross sections of the femur (compact and trabecular bone) after alizarin red S and H&E staining. The degree of calcification (intensity of red color) was visibly lower in the bone from the control group as compared to that in the CCN group (average 200.9 vs. 196.9), and the repeatability of measurements was significantly different ($p \leq 0.05$) (Figure 7). These results show greater amount of alizarin (positive calcified bone) in the CCN group, thus confirming better mineralization in this group.

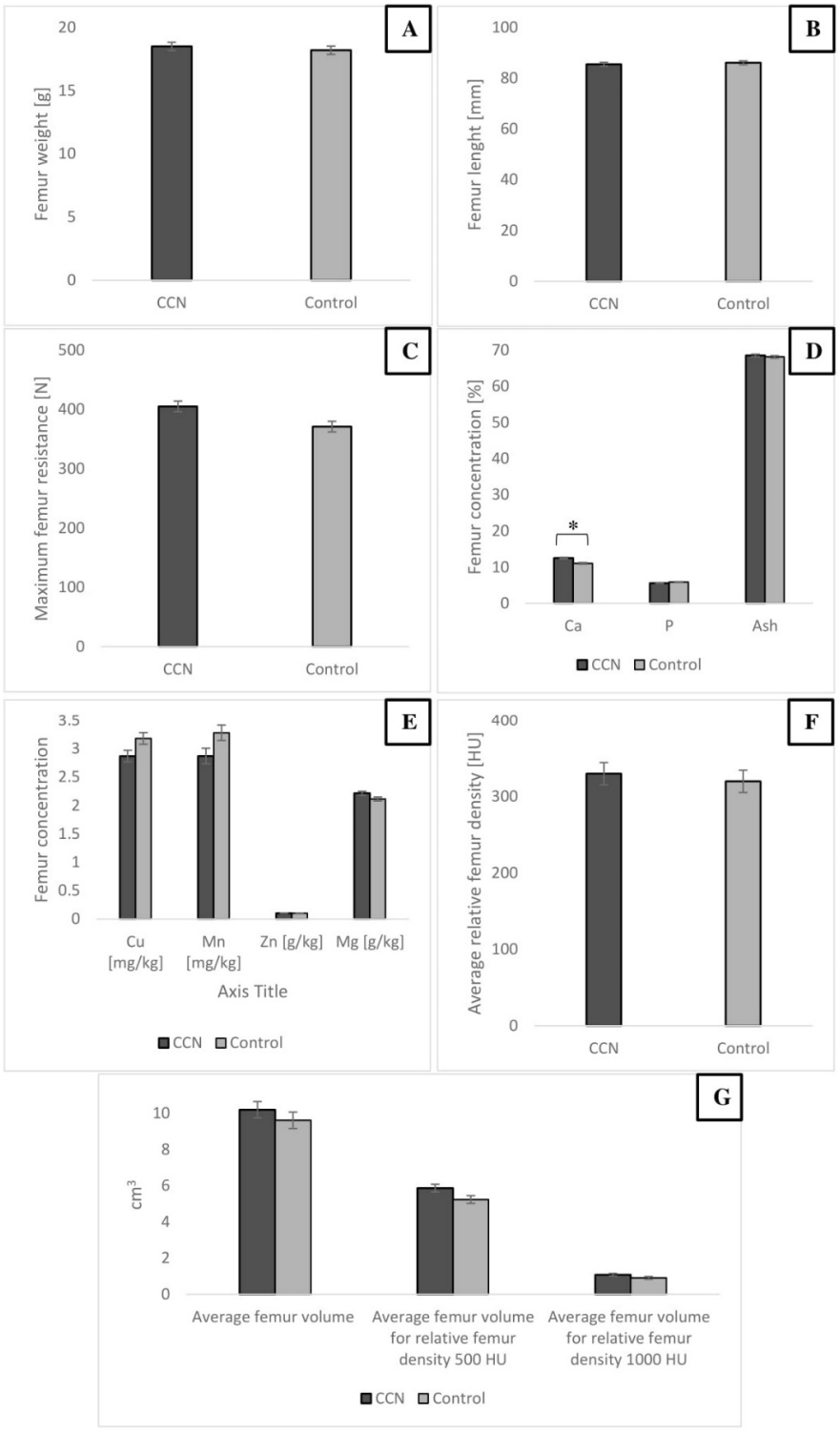

Figure 6. Selected parameters of broiler chicken male femoral bone from different groups. Femur weight (**A**), femur length (**B**), femur maximum resistance to breaking (**C**), femur Ca, P and ash content (**D**), femur micromineral content (**E**), femur average relative mineral density (**F**), average femur volume and average femur volume for 500 and 1000 HU (**G**). * Value on bars differs significantly at $p \leq 0.05$. The error lines represent standard error of mean.

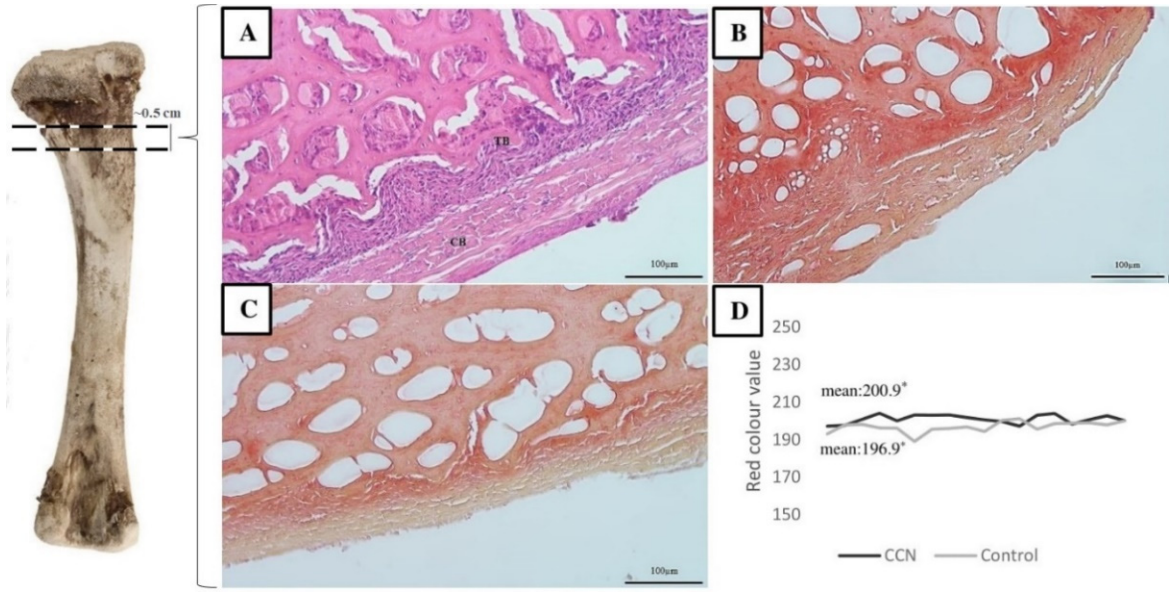

Figure 7. Histological cross sections from proximal metaphysis of broiler chicken male femoral bone. Femur after H&E staining (**A**). Alizarin red staining for mineralization (calcium deposits) for broiler chicken after IOI with CCN (**B**) and without IOI (**C**). Higher red colour intensity value suggests better calcification of the bone (**D**). TB: trabecular bone; CB: compact bone. * The value next to the averages differs significantly at $p \leq 0.05$.

3.5. Molecular Outcomes—OC Concentration in Serum and Femur of Embryo and Broiler Chicken

Figure 8 shows the concentration of OC in the serum and femur of embryos and broiler chickens. In embryos inoculated with CCN, the concentration of OC was lower in the serum but higher in the femur. In broilers, OC levels were not different in the serum; however, CCN birds showed a higher OC concentration in the femur compared to the control group ($p \leq 0.05$). OC concentration in bones suggests better mineralization.

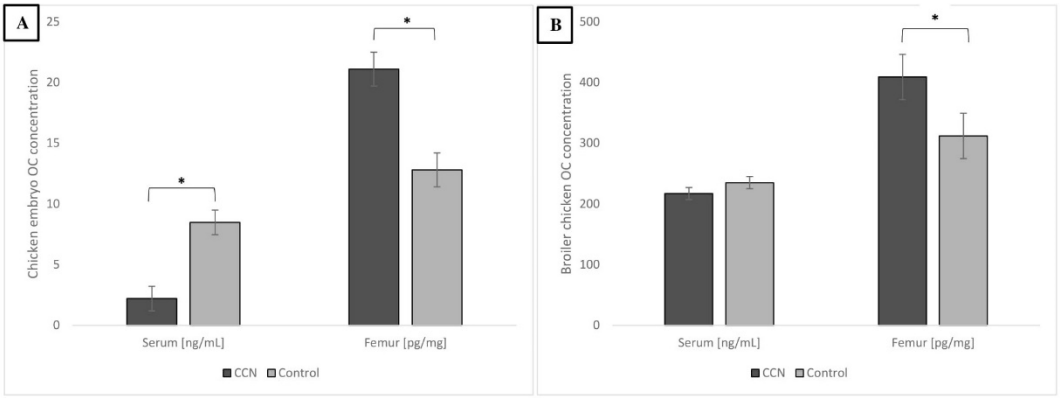

Figure 8. The OC concentration in serum and femoral bone from chicken embryo on 20 day after IOI with CNN (**A**) and from broiler chicken male after 42 d of rearing period (**B**). * Value on bars differs significantly at $p \leq 0.05$. The error lines represent SEM.

4. Discussion

Nanometric forms of minerals have a high potential to support growth at lower doses compared to conventional organic or inorganic sources of minerals, including Ca [38]. The application of nanotechnology to poultry has been receiving increasing interest because of the continuous search for alternative sources of macro and microminerals that could be more efficiently used in this field. Calcium nanoparticles and their application in poultry production have also become a hot topic of research in recent years [13,16,17,25,39–42].

Most of the studies have mainly focused on the administration of nanoparticles in water or feed to poultry to improve the health status of birds, productivity, and absorption of minerals. Our study is one of the few research studies investigating the effect IOI of Ca nanoparticles on the skeletal system of birds. The aspect of bird bone quality appears to be important while studying Ca nanoparticles.

The present study applied a high concentration of nanoparticles (500 µg/mL). The in vitro viability assay showed better viability of bone cells at the higher concentration of CCN. We assumed that the applied concentration would not be harmful but more conspicuous, in accordance with our pilot studies. Furthermore, based on our previous study [24], the potential effects of sham control inoculated with PBS were not evaluated in the present work, because of high mortality in the sham control group.

In our previous study [24], the effect of nano-hydroxyapatite (HA-NP) on chicken embryo development, particularly on the skeletal system, was evaluated. The results indicated that HA-NP did not negatively influence embryo development, but influenced molecular responses at the stage of embryogenesis, which were not reflected in bone development of the embryo. In the present study, we decided to use another type of Ca nanoparticles—CCN. The positive effect of IOI of CCN had been already demonstrated by Salary et al. [25] by using the highest concentration of nanoparticles at 200 µg/mL.

The hatchability results of chicken embryos were consistent with those of recent studies by Ahmadzadeh et al. and Matuszewski et al. [24,26] who investigated the use of HA-NP from different origins and CCN [25]. This suggests that CCN, similar to HA-NP, are safe and nonhazardous to the chicken embryo. However, it is worth noting that hatchability was generally lower in the study of Salary et al. [25] even though they used lower concentrations of CCN. The hatchability is influenced by the type of applicated nanoparticles, place and time of inoculation [24], and finally, by the hatching eggs quality [43]. Although the BW of the embryo was not affected, we observed an increase in liver weight. This observation is difficult to explain because of lack of similar studies on this topic; however, CCN may induce differential organ development of chicken embryo [44]. The use of dicalcium phosphate nanoparticles in broiler feed did not affect liver weight [41]. It should be noted that the weight of the bursa of Fabricius and spleen was significantly higher in broiler hatchlings after IOI of CCN in the study of Salary et al. [25]. Chicken embryos are highly sensitive models for testing potential toxic effects of the inoculated substances by monitoring total and differential organ development rates and survivability of the embryos. In the present study, no significant differences were observed in serum biochemical parameters, except MDA, which is a product of lipid peroxidation and Glu. Our results suggest higher lipid peroxidation in the control group; hence, the administration of CCN was found to decrease lipid peroxidation level (which should be considered as a positive effect). It is, however, quite difficult to interpret the relevance of decreased MDA content because of little understanding of the regulation of the antioxidant system in avian embryo. The hydrocolloids of CCN could additionally dilute egg content, resulting in lower lipid peroxidation in embryo. Nevertheless, blood biochemical indicators rarely reflect the actual status of the bird's health because of their conditioning by many other factors, and in this study, the values for both groups were within the range for poultry [45]. Furthermore, our numerical results agree with those of Ahmadzadeh et al. [26] who demonstrated higher values of AST and ALT in groups after IOI with chemically synthesized hydroxyapatite than in controls. The higher values could be automatically equated with metabolic and liver disfunctions or stress [46]; however, in the present study, we did not observe significant differences between the control and CCN groups. The form and size of the compound supplemented in ovo can affect the organ functions of the embryo. AST and ALT enzymes are normally found in hepatocytes. ALT is a more specific indicator of liver diseases, while AST is commonly found in other organs such as skeletal muscle [47]. In cases of bone metabolism disorders such as phosphatemia, which occur due to decreased ALP levels, bones are poorly mineralized. Thus, serum ALP levels are measured to diagnose bone mineralization and liver functions [48]. In our study, the CCN group showed higher ALP

concentration, but the difference was not significant. ALP concentration indicates better mineralization of bones, and results for IOI of CCN obtained by Salary et al. [25] confirmed our findings. Adequate situation was demonstrated according to bacterial synthesized ionic nano-hydroxyapatite applicated to the egg, which influenced more effectively bone mineral content and resulted in higher ALP values of the embryos' serum [26].

It is well known that bone development dynamics vary in different types of bones. The tibia and femur, for example, show different changes in their structural, mechanical, and compositional properties [49]. The IOI of minerals may affect bone properties. Oliveira et al. [50] showed that the application of organic zinc, copper, and manganese did not affect tibia measurements of 1-day-old hatchlings. Similar results were noted in our previous study—HA-NPs did not affect the width and length of the tibia but affected its weight [24]. The IOI of copper nanoparticles significantly increased femur weight and length in broilers [51]. Ca along with P are the main contributors to bone mineral structure. The mineralization of chicken bones affects their strength, which, in turn, is determined by the mass, volume, microarchitectural organization, and degree of mineralization of bone matrix [52]. The standard indicators of bone mineralization status include various measurements that are partly correlated. These measurements include breaking strength (or resistance to breaking) [53], mineral density [54], crude ash content [53,55], and elemental mineral content, including Ca, P, and microminerals [54]. All these measurements were performed in the present study. The results showed that bone measurements were not affected by IOI of CCN; however, the content of Ca and P in femur and tibia bones was significantly different between the embryo groups. Similar results were also reported by Salary et al. [25], where significant differences in Ca and P concentrations in broiler bones were observed after IOI with 100 and 200 µg/mL of CCN. Moreover, IOI of nutrients increased the Ca and P content of the tibia in embryos on the 19th day of incubation [56]. This is important because almost 99% Ca is derived from skeleton, where it forms hydroxyapatite together with P. In the present study, based on the observed bone quality parameters of broiler chickens, it could be stated that IOI of CCN at 500 µg/mL concentration may cause far-reaching effects—better calcification in broiler chicken's leg bones. The Ca content in broiler femur was significantly higher in the experimental group than in the control group. However, no differences were observed in the content of other microminerals (Zn, Mg, Mn, and Cu); this finding differed from that of Salary et al. [25] who demonstrated significantly higher Cu values in broiler hatchling bone after IOI of CCN. Increased Ca content may provide higher resistance of bone to breaking, although this aspect was not confirmed in the present study. In broiler chickens, the breaking strength of the femur bone plays an important role in deformities of skeletal system, because this bone is considered to support and perform weight-bearing functions [51]. The bone strength is determined by multiple factors, including bird's growth rate, age, sex, endocrinal metabolism, bird handling, and feeding [57]. Considering nutritional factors, it is reasonable to pay attention to in ovo feeding using different nutrients through inoculation to improve birds' bone quality.

The CCN group showed a tendency ($p = 0.053$) of the higher relative femur density compared to the control. This could suggest better mineralization of the femur and actually was reflected in higher Ca concentration in this study. Parameters such as relative bone density and bone volume can differ and depend on factors such as sex, breed, or strain of the chicken [58]. Male chickens are more susceptible to skeletal disorders, especially because they have higher weight gain. Moreover, differences in bone mineral density are influenced by the region where it is measured. In the present study, the measurements of diaphysis, proximal metaphysis, and distal metaphysis were separately performed, but not included. The average density of the entire bone was presented.

The intensity of red color after alizarin red S staining was higher in the femur of the CCN group. Alizarin red S is commonly used in histology and histopathology to stain or locate Ca deposits and Ca-binding proteins and proteoglycans in tissues [59] or cell cultures [60]. Studies on alizarin red S staining usually used rodents as a research model. For example, Fouad-Elhady et al. [36] applied alizarin red S staining to determine the

mineralization intensity of the femur bone in a rat model. The degree of calcification was markedly reduced in osteoporotic rats as compared to that in gonad-intact rats, and the maximum amount of alizarin red S (positive calcified bone) was observed in the group treated with HA-NP. The red values were higher in rats than in chicken used in this study.

A few studies have focused on IOI of nutrients in nanometric form and evaluated their effect on after-hatched chicken. In our study, we did not observe any negative effects of IOI of CCN on the final BW, FCR and mortality of chickens after 42 days of rearing. The physicochemical properties of breast muscle were also not affected, despite a change in the color parameter a*, which was probably due to other factors, such as the storage of birds after slaughter. Salary et al. [25] also did not show any significant effect on production results (feed intake, weight gain and FCR) in broilers at 1 to 21 days of age. Other studies on this aspect have been reported, but with IOI of other minerals in nano form. For example, Mroczek-Sosnowska et al. [23] showed the positive influence of Cu nanoparticles and $CuSO_4$ on broiler chicken performance. At the end of the rearing period (day 42), the BW was significantly higher in the NanoCu and $CuSO_4$ groups than in the control group (2000 g in control vs. 2206 g in NanoCu and 2402 g in $CuSO_4$ groups). Both treatment groups had significantly lower FCR and mortality and higher percentage of breast and leg muscles in the carcass than the control group. Several studies have addressed the issue of per os feeding with calcium compound nanoparticles and showed measurable benefits of their use in poultry nutrition. Samanta et al. [39] demonstrated better growth performance in broilers fed calcium phosphate nanoparticles at 50% level. The inclusion of HA-NP in chicken diet led to improved BW gain and feed intake, while Ca and P from HA-NP were better absorbed by birds [42]. Additionally, higher final BW, better FCR, and higher daily weight gains were observed in groups of birds fed diets with nano dicalcium phosphate [41].

Bone modeling is defined as either the formation of bone by osteoblasts or resorption of bone by osteoclasts on a given surface. This contrasts with bone remodeling, in which osteoblast and osteoclast activities occur sequentially in a coupled manner on a given bone surface [61]. The imbalance between these processes might led to the occurrence of osteoporosis—a disease characterized by loss of mineralized structural bones [62,63]. Bone modeling is strictly associated with biochemical bone turnover markers, which play different roles in organisms and are often specific to bone tissue. OC is a major, noncollagenous protein in bones. Recently, this protein has been used as a biomarker of osteoblast activity for evaluating bone remodeling [64]. Few studies have investigated the use of OC as a bone turnover marker in birds. It has been shown that serum OC concentration in laying hens decreases with age. The OC increased in the experimental group (fed with high energy and low protein diet) suggesting greater bone turnover. The quality of bones was not improved, even aggravating the incidence of skeletal damage [65]. Both Ca and P deficiencies reduce hydroxyapatite crystal formation. When bone mineralization decreases, free OC may be available for circulation in the blood [66]. On the other hand, higher OC levels, especially the carboxylated form of OC, may suggest better mineralization process. This dependence was verified in our study, because better mineralization (especially Ca) of bone was observed in embryo and chicken. The CCN group showed higher OC in embryo femur, whereas the control group showed higher concentration of OC in serum suggesting greater bone turnover in control group. In adult birds, serum OC level was similar, but higher in the femur of the CCN group. Because of its nanometric size and easy uptake by cells during embryogenesis, CCN may effectively modulate bone mineralization. Nanoparticles as an external source of Ca allow to reduce the process of hydroxyapatite biosynthesis in osteoblasts and may affect the regulation of bone OC—the protein responsible for hydroxyapatite binding, ultimately resulting in an increase in bone mineralization. This modulation can provide a sustained effect, thereby improving bone quality, even in adult birds. However, further research on this topic is needed.

5. Conclusions

It can be concluded that CCN are biocompatible and osteoconductive nanoparticles. The IOI of CCN at the concentration of 500 µg/mL was not harmful to chicken embryos and did not affect hatchability, BW, or muscle weight of the embryos but affected their liver weight. No negative effects were observed for serum biochemical parameters. Moreover, Ca and P content increased in embryo femur and tibia in CCN group. IOI with 500 µg/mL concentration of CCN did not negatively influence the production results and health of the birds from day 1 to day 42 of rearing. The slaughter analysis showed that meat quality (except color parameter a*) of male carcasses was also not affected. Ca concentration in the femur bone of broiler chickens was significantly higher in the CCN group than in the control group, which was a positive aspect. The study did not show any significant differences in bone weight, bone length, and micromineral content in the femur as well as in the scanning results of broiler chickens' femur. The degree of calcification (intensity of red color) was visibly lower in the bone from the control group than in the CCN group, and the repeatability of measurements was significantly different. The OC concentration in CNN embryos was lower in serum but higher in femur than in the control group. In broilers, CNN group increased OC in femur. The IOI of CCN could modify the molecular responses at the stage of embryogenesis, resulting in better mineralization and could even provide a sustained effect, thereby improving bone quality in adult birds through the calcification of the femur. It can be concluded that IOI with CCN at the concentration of 500 µg/mL did not cause harmful effects and can be used as an alternative method to standard feeding, for improving bone quality of broiler chickens. Further research is needed to determine the possibility of reducing in-feed Ca inclusion in response to IOI of CCN.

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Warszawa, 12.05.2021 r.

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W związku z ubieganiem się mgr. inż. Arkadiusza Matuszewskiego o stopień doktora nauk rolniczych oświadczam, że w pracy pt. „Calcium and phosphorus and their nanoparticle forms in poultry nutrition" Matuszewski A., Łukasiewicz M., Niemiec J. World's Poultry Science Journal. 2020, 76(2), 328–345 mój udział polegał na pomocy w przygotowaniu tekstu publikacji i przy recenzji. Udział procentowy szacuję na 10%.

Łukasiewicz-Mierzejewska M.
(czytelny podpis)

Warszawa, 12.05.2021 r.

Imię i nazwisko
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(czytelny podpis)

Warszawa, 12.05.2021 r.

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(czytelny podpis)

Warszawa, 12.05.2021 r.

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(czytelny podpis)

Warszawa, 12.05.2021 r.

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Jaworski
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(czytelny podpis)

Warszawa, 12.05.2021 r.

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(czytelny podpis)

Warszawa, 12.05.2021 r.

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Szudrowicz Hubert
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(czytelny podpis)

Warszawa, 12.05.2021 r.

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(czytelny podpis)

Warszawa, 12.05.2021 r.

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A. Chwalibog

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(czytelny podpis)

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Ewa Sawosz
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(czytelny podpis)

Warszawa, 12.05.2021 r.

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Łukasiewicz-Mierzejewska M.

(czytelny podpis)

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Jaworski

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(czytelny podpis)

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(czytelny podpis)

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A Chwalibog

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(czytelny podpis)

Warszawa, 12.05.2021 r.

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